Universidade de Lisboa
Faculdade de Medicina

STUDIES ON A HUMAN MODEL AND A MURINE MODEL OF IMMUNODEFICIENCY: HIV-2 INFECTION AND TACI DEFICIENCY.

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Doutoramento em Ciências Biomédicas
Especialidade de Ciências Biopatológicas

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Tese orientada pela Prof. Doutora Ana Espada de Sousa
e pela Prof. Doutora Marília Cascalho

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Mantchev G, Cortesão CS*, Rebrovich M, Cascalho M, Bram R.* TACI is required for efficient plasma cell differentiation in response to T-independent type 2 antigens. *J Immunol 2007; 179:2282-8.*
* GM e CSC contribuíram igualmente.

Gautier D, Beq S, Cortesão CS, Sousa AE, Cheynier R. Efficient thymopoiesis participates in the maintenance of peripheral CD4 T-cells during chronic HIV-2 infection. *J Virol. 2007; 81(22):12685-8.*

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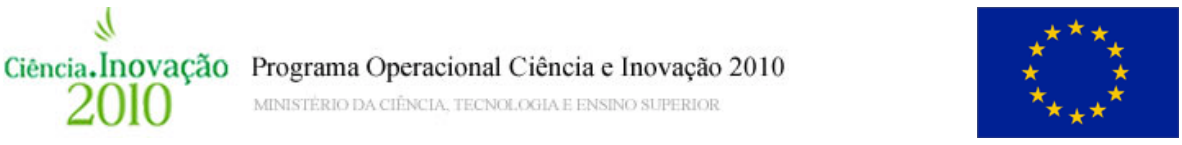

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Abbreviations

γc	Common Cytokine Receptor Chain
[3H]TdR	Tritiated Thymidine
2-ME	2 - Mercaptoethanol
Ab(s)	Antibody(ies)
Ag(s)	Antigen(s)
agm	African Green Monkeys
AICD	Activation Induced Cell Death
AIDS	Acquired Immunodeficiency Syndrome
AP-1	Activator Protein 1
APRIL	A Proliferation Inducing Ligand
ART	Antiretroviral Therapy
ASCs	Antibody-Secreting Cells
BAFF	B Cell Activating Factor
BAFF-R	B Cell Activating Factor Receptor
Bcl-2	B-Cell Lymphoma 2
BCL-6	B Cell Lymphoma 6
BCMA	B Cell Maturation Antigen
BCR	B Cell Receptor
Blimp-1	B Lymphocyte-Induced Maturation Protein-1
BLyS	B Lymphocyte Stimulator (Also Known As BAFF)
BSA	Bovine Serum Albumin
CAML	Calcium Modulating Cyclophilin Ligand
CCL	Chemokine (C-C motif) Ligand
CCR	Chemokine (C-C motif) Receptor
CD	Cluster of Differentiation
CDC	Centers For Disease Control and Prevention
CDR	Complementarity Determining Region
CLP	Common Lymphoid Precursors
CRDs	Cysteine-Rich Domains
CVID	Common Variable Immunodeficiency
CXCL	Chemokine (CXC motif) Ligand
CXCR	Chemokine (CXC motif) Receptor
DN	Double-Negative
DNA	Deoxyribonucleic Acid
DP	Double-Positive
ELISA	Enzyme-Linked Immunosorbent Assays
ELISPOT	Enzyme-Linked Immunosorbent Spot Assay
FCS	Fetal Calf Serum
FITC	Fluorescein
GALT	Gut-Associated Lymphoid Tissue
gp	Glycoprotein
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HTLV-III	Human T-Lymphotropic Virus Type III
IDCV	*Imunodeficiência Comum Variável*
Ig	Immunoglobulin

IL	Interleukin
IL-7	Interleukin 7
IL-7Rα	Interleukin 7 Receptor α (CD127)
InsP3	Inositol-1,4,5-Trisphosphate
JNK	c-Jun NH2-Terminal Kinase
KO	Knockout
LAV	Lymphadenopathy-Associated Virus
LPS	Lipopolysaccharide
LTNP	Long-Term Non-Progressors
LTRs	Long Terminal Repeat Sequences
MHC	Major Histocompatibility Complex
MIP-1α	Macrophage Inflammatory Protein 1α
MIP-1β	Macrophage Inflammatory Protein 1β
Neu5Ac	N-Acetylneuraminic Acid
Neu5GC	N-Glycolylneuraminic Acid
NFAT	Nuclear Factor of Activated T Cells
NF-κB	Nuclear Factor Kappa B
NP	4-Hydroxy-3-Nitrophenylacetate Acid
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
Pen/Strep	Penicillin Streptomycin
PerCP	Peridinin chlorophyll
PICs	Preintegration Complexes
PLC-γ	Phospholipase C-γ
PNA	Peanut Aglutinin
QM	Quasi-Monoclonal
RANTES	Regulated Upon Activation, Normal T Cell Expressed And Secreted
rhIL-7	Recombinant Human IL-7
RNA	Ribonucleic Acid
RTE	Recent Thymic Emigrants
SDF-1	Stromal Derived Factor 1
SIDA	*Síndrome da Imunodeficiência Adquirida*
SIV	Simian Immunodeficiency Virus
sj	Signal Joint
sm	Sootey Mangabeys
TACI	Transmembrane Activator Calcium Modulator And Cyclophilin Ligand Interactor
TCR	T Cell Receptor
TI-2	T-Independent Type Ii
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
TRAFs	TNF Receptor Associated Factors
TREC	T Cell Receptor Excision Circles

Preface

My main scientific area of interest is lymphocyte biology specifically in the settings of immunodeficiencies. I started working in human T cells at the *Unidade de Imunologia Clínica* of the *Instituto de Medicina Molecular* with Prof. Doutora Ana Espada de Sousa, where I had the opportunity to develop a project on T cell production during HIV-2 infection. This project focused on a natural model of attenuated HIV disease and generated important results on the role of IL-7 and the contribution of the thymus for the understanding of HIV/AIDS pathogenesis that were published in:

"Rate of increase in circulating IL-7 and loss of IL-7Ralpha expression differ in HIV-1 and HIV-2 infections: two lymphopenic diseases with similar hyperimmune activation but distinct outcomes."(*J.Immunol 2007, 178, 3252-9*).

"Efficient thymopoiesis contributes to the maintenance of peripheral CD4 T cells during chronic human immunodeficiency virus type 2 infection". (J.Virol 2007, 81, 12685-8).

After developing this study in human immunodeficiency, I was offered the possibility to develop research in a murine model of B cell immunodeficiency, at the Transplantation Biology Unit, Mayo Clinic, with Prof. Doutora Marília Cascalho. The work done with a murine model of TACI knockout generated data that contributed to the understanding of the role of TACI in the differentiation of plasma cells upon antigenic stimulation, and resulted in the publication:

"TACI is required for efficient plasma cell differentiation in response to T-independent type 2 antigens."(J Immunol 2007, 179, 2282-8).

Thus, the present thesis is organized into two Parts. Part I is entitled "T-cell production in HIV-2 infection: insights to AIDS pathogenesis" and Part II is entitled "TACI regulation of B cell differentiation after antigenic stimulation". Given the distinct topics studied, each part includes an Introduction / State of the Art, a Result section comprising the published papers and a section summarizing the Conclusions and Future Perspectives.

In conclusion, it is clear to me that these two different experiences during my Ph.D. were critical for my development as a scientist. I had the opportunity to work in basic and clinical science, in two completely different systems, human studies and mouse knockout models, which allowed me to learn an array of different techniques. Also, it has brought me closer to different forms of scientific reasoning, as well as new ways of posing scientific questions and design of experiments to answer them.

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Catarina dos Santos Cortesão

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I have already started working on a very promising scientific adventure at the Epigenetics and Soma Lab (*Instituto Gulbenkian de Ciência*) and I want to thank Vasco Barreto for his support and patience and my co-workers Clara Pereira, Nadine Caratão, Daniel Espadinha and Thiago Guzella for their help in these transition times.

And furthermore, since not all of my time was spent doing science, I want to thank all my friends from around the world that helped me keep my sanity and made my life so much better.

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Para o Rui

Para os meus Pais e Irmão

Resumo

Neste trabalho foram investigados alguns mecanismos que contribuem para a manutenção de imunocompetência usando dois modelos: um modelo humano natural atenuado da síndrome da imunodeficiência adquirida (SIDA), a infecção pelo HIV-2; e um modelo murino de deficiência de células B por defeito de TACI (*Transmembrane Activator and Calcium Modulating Cyclophilin Ligand Interactor*).

A Parte I deste trabalho intitulada "Produção de células T durante a infecção pelo HIV-2" teve por objectivo estudar o impacto da infecção pelo Vírus da Imunodeficiência Humana tipo 2 (HIV-2) na maturação das células T no timo e na sua diferenciação na periferia. A epidemia HIV/SIDA constitui actualmente um dos principais problemas de saúde, estimando-se que entre 1981 e 2007 aproximadamente 65 milhões de pessoas foram infectadas em todo o mundo. Os agentes responsáveis pela SIDA são o HIV-1 e HIV-2. A infecção por HIV-1 é caracterizada por uma inexorável depleção das células T CD4, para a qual contribuem vários factores além de efeitos citopáticos do vírus. Independentemente dos mecanismos envolvidos na depleção das células T CD4, um factor determinante do ritmo de progressão da doença é a capacidade de reconstituição do compartimento de células T. Num indivíduo saudável, a manutenção da população de células T é assegurada por mecanismos homeostáticos que regulam a proliferação e sobrevivência de células na periferia, conjuntamente com a produção de linfócitos no timo, sendo a contribuição tímica dependente da idade. A infecção pelo HIV-1 associa-se a alterações da produção *de novo* de células T e, por outro lado, a uma activação imunitária persistente que compromete os mecanismos de proliferação homeostática. Na infecção por HIV-2, a progressão da doença é mais lenta do que por HIV-1 associando-se a um impacto limitado na sobrevivência da maioria dos adultos infectados. A infecção por HIV-2 caracteriza-se por um ritmo de perda das células T CD4 menos acentuado e por menor carga viral circulante, embora os níveis de activação imunitária sejam semelhantes nos doentes infectados pelo HIV-1 e pelo HIV-2 com grau de depleção de células T CD4 comparável. Deste modo, a infecção por HIV-2 pode ser considerada um modelo natural de doença HIV "atenuada".

O primeiro objectivo deste trabalho foi estudar o papel da interleucina 7 (IL-7), uma citocina essencial para a homeostasia das células T, em situações clínicas de linfopenia provocadas pela infecção pelo HIV-2. Os níveis séricos de IL-7 foram quantificados por ELISA e determinou-se a expressão da cadeia α do seu receptor (IL-7Rα) em células T CD4 e CD8

por citometria de fluxo, num estudo transversal envolvendo grupos de doentes infectados pelo HIV-2 e doentes infectados pelo HIV-1, equiparados para idade e estadio da doença. Os resultados documentaram uma correlação entre os níveis séricos de IL-7 e o grau de depleção de células T CD4 na infecção pelo HIV-2, que se revelou significativamente mais forte do que a observada na infecção pelo HIV-1, sendo esta semelhante à documentada em outros *cohorts* de doentes infectados pelo HIV-1. É importante realçar que na fase inicial da infecção por HIV-2 os níveis séricos de IL-7 não diferem do grupo controlo de indivíduos saudáveis, ao contrário dos níveis elevados documentados nos doentes infectados pelo HIV-1. Nos estadios mais avançados, os níveis séricos de IL-7 apresentam-se significativamente aumentados em ambas as infecções em comparação com o grupo de controlos saudáveis. Em relação ao receptor IL-7Rα documentou-se uma melhor preservação da sua expressão nas diferentes sub-populações de células T, em particular nas células T CD4 *naive*, nos doentes infectados pelo HIV-2. Estes resultados sugerem uma maximização da utilização dos recursos de IL-7 disponíveis durante a infecção pelo HIV-2.

O timo é essencial para o desenvolvimento e maturação das células T, contribuindo assim para manter a diversidade do repertório do receptor das células T (TCR). Esta diversidade é considerada fundamental para montar respostas contra novos agentes patogénicos e para prevenir a evasão de microorganismos persistentes às respostas do sistema imunitário. Apesar de o timo involuir com a idade, é actualmente reconhecida a funcionalidade do timo adulto, principalmente em situações clínicas em que há necessidade de reconstituir as populações de células T na periferia. Não existem estudos sobre a função tímica na infecção pelo HIV-2. É plausível admitir que o melhor curso da infecção pelo HIV-2 seja devido, em parte, a uma melhor capacidade do timo de compensar a perda de células T e de manter na periferia uma maior diversidade do repertório do TCR. A função tímica pode ser avaliada através da quantificação de pequenos fragmentos de DNA epissómico designadas *T-cell receptor excision circles* (TREC), resultantes dos rearranjos, na timopoiese, dos genes das cadeias que compõem o TCR. Durante a maturação das células T αβ, ocorre o rearranjo do gene da cadeia β seguido do rearranjo do gene da cadeia α, originando, respectivamente, βTREC e sjTREC entre outros. A proliferação celular que ocorre entre estes dois rearranjos é o principal determinante do número de timócitos gerados no timo. Como os níveis de TREC se diluem progressivamente com a proliferação celular, a quantificação do rácio sj/β TREC nas células do sangue periférico permite estimar a dinâmica proliferativa intratímica e, desta

forma, indirectamente a produção de linfócitos T no timo. Assim, no segundo objectivo proposto para esta parte do trabalho, utilizou-se esta metodologia para estimar a função tímica em grupos de doentes infectados com HIV-2 e doentes infectados com HIV-1, correlacionando-a com parâmetros de progressão da doença. Tal como esperado, os indivíduos saudáveis apresentaram uma diminuição do rácio sj/βTREC de acordo com a idade, sendo estes valores significativamente inferiores na infecção pelo HIV-1. Nos doentes infectados pelo HIV-2 documentou-se uma relativa manutenção do rácio sj/β TREC com a idade.

Em conclusão, os resultados aqui apresentados sugerem que na infecção pelo HIV-2, quer a timopoiese quer a homeostasia das células T na periferia estão menos comprometidas, podendo contribuir para um ritmo de progressão da doença mais lento. Estes resultados enfatizam o papel do timo como alvo terapêutico para uma completa reconstituição imunitária em conjugação com terapêutica anti-retroviral na infecção pelo HIV-1.

A Parte II deste trabalho, intitulada "Regulação pelo TACI da diferenciação de células B após estimulação antigénica", teve por objectivo geral clarificar o papel da molécula TACI na diferenciação de células B. Esta molécula é uma proteína transmembranar tipo III da superfamília dos receptores do TNF (*Tumor Necrosis Factor*), expressa em células B. A sua importância tem sido evidenciada em estudos de modelos de ratinho com deficiência em TACI onde se observam infecções bacterianas recorrentes do tracto respiratório e hipogamaglobulinemia, designadamente de IgG e IgA, atribuídas a uma disfunção das células B. A deficiência em TACI foi recentemente associada a Imunodeficiência Comum Variável (IDCV), um grupo heterogéneo de doenças caracterizadas por deficiência de produção de anticorpos. Estima-se que 5% a 10% dos doentes diagnosticados com IDCV têm pelo menos uma mutação no *locus TNFRSF13B* que codifica o TACI.

Com o objectivo de clarificar de que forma o TACI promove a produção de anticorpos, examinou-se o efeito da deficiência de TACI num modelo de ratinho em que a maioria das células B produz um anticorpo especifico para o hapteno *4-hydroxy, 3-nitro-phenylacetic acid* (NP), o modelo Quasi-Monoclonal (QM). Os ratinhos QM TACI KO e QM TACI+ foram imunizados com NP-Ficoll, que activa as células B na ausência de ajuda das células T e por essa razão se denomina um estimulo T-independente. A frequência de células B

produtoras de anticorpos foi avaliada por ELISPOT e os anticorpos produzidos foram quantificados por ELISA. Neste ratinho observou-se que a deficiência em TACI nas células B causa uma diminuição no numero de células produtoras de IgM e IgG específicas para NP em resposta a NP-Ficoll. Determinou-se que a origem do defeito na produção de anticorpos reside no estadio precedendo a diferenciação em plasmócitos. De facto, deficiência em TACI não diminui a intensidade e frequência de activação ou proliferação das células B quando estimuladas por NP-Ficoll. Pelo contrário, células B deficientes em TACI permaneceram em ciclo celular durante mais tempo e produziram clones maiores do que células B TACI+ após imunização com NP-Ficoll sugerindo controle da expansão clonal por TACI.

Estes resultados sugerem que TACI exerce duas funções autónomas nas células B: por um lado, controla o número de ciclos de proliferação após activação; por outro lado, estimula a diferenciação das células B em plasmócitos. Assim, este estudo contribuiu para clarificar o aparente paradoxo que envolve o papel simultaneamente activador e inibidor do TACI na função das células B.

Palavras Chave: HIV/AIDS; HIV-2; IL-7/IL7R-α; Timo; TREC; TACI; respostas de células B independentes de T; diferenciação de plasmócitos.

Summary

This work aimed to investigate mechanisms that contribute to the maintenance of immune competence using two models: the HIV-2 associated immunodeficiency and a murine model of B cell immunodeficiency, the TACI (Transmembrane Activator and Calcium Modulating Cyclophilin Ligand Interactor) deficiency.

Part I, entitled "T cell production in HIV-2 infection: insights to AIDS pathogenesis", was designed to study the impact of infection with HIV-2 on T cell maturation in the thymus and on their differentiation in the periphery in order to provide insights into HIV/AIDS pathogenesis. AIDS epidemic represents one of the most important health problems world-wide. Between 1981, when the first AIDS patients were described, and 2007, approximately 65 million cases of HIV infection have been diagnosed and 25 million people have died of AIDS-related illnesses. The viral agents responsible for AIDS are HIV-1 and HIV-2. HIV-1 infection is characterized by a progressive and relentless decline of CD4 T cells and this depletion cannot be solely attributed to the directed effects of cell infection by the virus *per se*. Regardless of the mechanisms involved in CD4 T cell depletion, the replenishment of the T cell pool is a major determinant of disease progression. In a healthy state, the maintenance of the naive T cell pool is currently thought to be due to homeostatic mechanisms that control proliferation and survival of peripheral T cells combined with an age-dependent contribution of thymic output. Importantly, *de novo* T cell production in the thymus is impaired by HIV-1 infection. Additionally, the HIV-associated persistent hyper-immune activation is known to increase susceptibility to apoptosis and to impair peripheral T cell proliferation as well as to increase the outflow of naive T cells into the memory-effector compartment. HIV-2 infection is characterized by a progressive CD4 T cell decline that is much slower than in HIV-1 infection, but features similar levels of hyper-immune activation throughout the natural history of the disease, despite the reduced viremia. Therefore, HIV-2 was used in this work as a model of "attenuated" HIV disease to provide insights into the alterations of the mechanisms necessary for the replenishment of the T cell pool during HIV/AIDS pathogenesis.

The first objective was to address the role of interleukin 7 (IL-7), a nonredundant cytokine for T cell homeostasis, in lymphopenic clinical settings associated to HIV-2. It has been previously shown that IL-7 levels correlate with CD4 T cell depletion during HIV-1 infection. Serum IL-7 was quantified by ELISA and the expression of the α chain of the IL-7

receptor (IL-Rα) in CD4 and CD8 T cells was assessed by flow cytometry, in a cross-sectional study involving HIV-2 and HIV-1 cohorts of patients paired for disease stage. A strong positive correlation was documented between circulating IL-7 levels and the degree of CD4 T cell depletion in HIV-2 infection. Moreover, in contrast to HIV-1 infection, the levels of circulating IL-7 were not significantly increased in the early stages of HIV-2 infection. On the other hand, at advanced stages, similar levels of circulating IL-7 were found in both HIV-2 and HIV-1 cohorts, which were significantly higher than the ones found in seronegative controls. In relation to the IL-7 receptor, a clear better preservation of IL-7Rα expression was documented in different lymphocyte T sub-populations in the HIV-2 infected cohort, in particular within the naive CD4 T cell pool. Overall, the data suggest a maximization of available IL-7 resources in HIV-2 infection.

The thymus provides a unique environment for T cells to mature and to generate a highly diverse repertoire of T cell receptors (TCR). Diversity of the TCR repertoire is considered necessary for recognition of all possible "invaders" and for mounting adequate specific responses to persistent pathogens in order to prevent escape. There is increasing evidence for the functional capacity of the adult thymus, particularly in clinical settings in which the T cell pool needs to be regenerated, despite thymic involution with age. There are no studies on thymic function in HIV-2 infection. It is conceivable that a better ability of the thymus to compensate the loss of naive T cells and to maintain a diverse TCR repertoire could contribute to the distinct course of the HIV-2 disease. T cell receptor excision circles (TREC) are small episomal DNA molecules excised from the genome of T cells during the rearrangement of their TCR in the thymus. The rearrangements of the *loci* of β and α chains of the TCR occur sequentially, generating βTREC and sjTREC, respectively. The cellular proliferation that occurs between these two recombination events is a major determinant of thymic output. Since TREC levels are diluted down during cell proliferation, the quantification of the ratio between β and sj TREC in peripheral cells gives an estimative of the intrathymic cellular division history that correlates with thymic output.

The second objective was to estimate thymic activity by measuring intrathymic proliferation history of circulating lymphocytes through the quantification of sj/β TREC ratio in order to evaluate a possible thymic contribution to the long term maintenance of peripheral CD4 T cells during HIV-2 infection. β and sj TREC levels were quantified in peripheral blood T

cells from HIV-2 and HIV-1 chronically infected patients without antiretroviral treatment. In seronegative controls there was a reduction in the sj/β TREC ratios with age in agreement with the expected thymic involution. The HIV-2 cohort presented elevated sj/β TREC ratios as compared to the ones found in HIV-1, suggesting a maintained thymic production for prolonged periods of time. In agreement, sj/β TREC ratios showed no significant reduction in older as compared to younger HIV-2 infected individuals.

Taken together, these data suggest that in HIV-2 infection, both thymopoiesis and peripheral T cell homeostasis are better preserved. Thus, during the course of HIV-2 infection there may be an apparent maintenance or possible rebound of thymic function that likely contributes to the more benign outcome of the infection. These results support the importance of the thymus as a target for immune-based interventions in order to achieve a complete immunologic reconstitution under antiretroviral therapy in HIV-1 disease.

The Part II of this work is entitled “TACI Regulation of B cell Differentiation after Antigenic Stimulation” and its main objective was to clarify the role of TACI in B cell differentiation. TACI deficiency leads to recurrent bacterial infections, particularly of the respiratory tract, and impaired responses to vaccination against encapsulated bacteria such as *Streptococcus pneumoniae* or *Haemophilus influenzae*. The impaired clearance of encapsulated bacteria in TACI deficient subjects has been attributed to reduced IgG and IgA production owing to a B cell defect. Also TACI deficiencies are associated with CVID in humans. CVID is a primary immunodeficiency that comprises a heterogeneous group of diseases with deficient antibody production. It is the most prevalent human primary immunodeficiency with clinical expression, affecting 1 in 25,000 Caucasians. Recent data showed that 5% to10% of subjects with CVID carry at least one germline mutation in the TACI *locus*. Thus, the second part of this work aimed to better understand how TACI promotes antibody production.

In order to clarify the role of TACI in B cell differentiation, TACI KO mice were bred with Quasi-Monoclonal (QM) mice that produce robust polysaccharide responses to 4-hydroxy, 3-nitro-phenylacetic acid (NP)-Ficoll, owing to the high precursor frequency of NP-specific B cells in the marginal zone of the spleen, to produce QM TACI KO mice. These mice were immunized with NP-Ficoll. The frequencies of antibody secreting B cells were measured by ELISPOT and the amount of antibodies produced was quantified by ELISA. It was observed

that QM TACI KO mice produce decreased numbers of IgM (2-fold) and IgG (1.6-fold) NP-specific antibody-secreting cells compared with QM TACI+ mice in response to immunization with NP-Ficoll. Moreover, these data indicated that TACI acts at a remote time from activation because TACI was shown not to be necessary for activation and proliferation of B cells both *in vitro* and *in vivo*. Instead, the results showed that QM TACI KO B cells remained in the cell cycle longer than QM TACI+ cells and had impaired plasma cell differentiation in response to NP-Ficoll.

These data indicated that TACI has dual B cell-autonomous functions, inhibiting prolonged B cell proliferation and stimulating plasma cell differentiation, therefore resolving the longstanding paradox that TACI may have both B cell-inhibitory and -stimulatory functions. By promoting plasma cell differentiation earlier during clonal expansion, TACI may decrease the chances of autoantibody production by somatic hypermutation of immunoglobulin genes in response to T-independent antigens.

Keywords: HIV/AIDS; HIV-2; IL-7/IL7R-α; Thymus; TREC; TACI; T-independent-B-cell responses; plasma cell differentiation.

PART I

T CELL PRODUCTION IN HIV-2 INFECTION: INSIGHTS TO AIDS PATHOGENESIS

"The more we learn about the world and the deeper our learning, the more conscious, specific and articulate will be our knowledge of what we do not know, our knowledge of our ignorance" **Sir Karl Popper**

Introduction

The Human Immunodeficiency Virus type 1 (HIV-1) was first isolated and characterized in the early 1980's, by two independent research teams. This virus, denominated lymphadenopathy-associated virus (LAV) by Luc Montagnier and colleagues (1) and termed human T-lymphotropic virus type III (HTLV-III) by Robert Gallo and colleagues (2), was independently isolated from a lymph node of a patient with lymphadenopathy, and from plasma of patients presenting with opportunistic infections or tumors (3-6). The HTLV-III/LAV virus, later termed HIV-1 (7), was found to be the causative infectious agent of this previously unknown acquired immunodeficiency syndrome (AIDS) in 1983 (8-10).

Between 1981, when the first AIDS patients were described, and 2007, approximately 65 million cases of HIV infection have been diagnosed and 25 million people have died of AIDS-related illnesses (11). Moreover, it is estimated that the number of new infections in 2007 were around 2.5 million cases and that over 5700 people die every day from AIDS (depicted in *Figure 1*).

The hallmark of HIV-1 infection is a profound immunodeficiency, associated with a progressive depletion and qualitative defects of the CD4 T cell subset, which in turn leads to failure of responses to opportunistic infections, followed by AIDS and death.

Soon after the first AIDS virus was described, Montagnier's research group described a second retrovirus linked to AIDS, subsequently termed HIV type 2 (HIV-2) (12). This new virus was isolated from West African patients with AIDS or AIDS-related symptoms (12, 13) and found to be quite common in the West African region (12, 14-17). Nowadays, HIV-2 still remains highly confined to West Africa (18), although HIV-2 infected cases can be found in certain European countries that have socio-economic ties with this area (19, 20). Portugal represents the only non-African country with a significant prevalence of HIV-2 due to connections with its ex-colonies. From a total of approximately 30.000 AIDS reported cases in Portugal, 3.4% are due to HIV-2 infection (21). In one study, more than 60% of all newly diagnosed HIV-2 infections in Portugal could not be directly traced back to a West African contact, demonstrating its entrenchment in the local population (22).

As described for HIV-1 infected individuals, CD4 T cell counts in HIV-2 infection decline progressively, although the disease progression is slower, leading to a limited impact on the survival of the majority of infected adults (23, 24). Importantly, viremia is substantially reduced in HIV-2 infection (25, 26). Thus, the study of HIV-2 infection offers the possibility to reassess the relative importance of virological and immunological parameters in HIV pathogenesis, by studying a human immunodeficiency virus that is associated with a clinically attenuated infection. The potential of the HIV- 2 model is yet to be fully explored, because of the geographic confinement of the infection to West Africa (27-29).

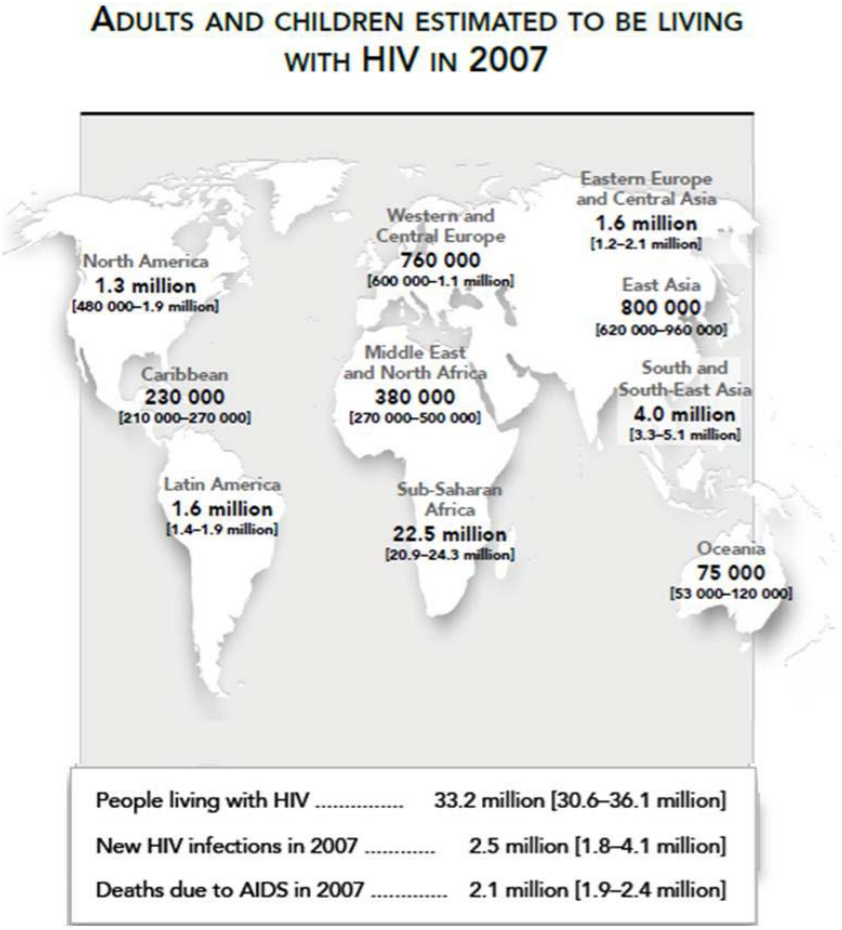


Figure 1 – Adults and children estimated to be living with AIDS.

The ranges around the estimates in the table define the boundaries within which the actual numbers lay, based on the best available information. Adapted from UNAIDS, AIDS epidemic update 2007 (11).

The Human Immunodeficiency Virus (HIV)

Origins of the virus

Evidence from genome sequencing suggests that HIV-1 and HIV-2 probably crossed the interspecies barrier from nonhuman primate species on several occasions (30, 31). The three genetic HIV-1 groups – M, N and O – resulted from independent events of cross specie transmission most likely from the chimpanzee *Pan troglodytes* (31-33). Of these events, only the M group with its numerous clades or subtypes (A to K) as well as circulating recombinant forms gave rise to the AIDS pandemic (34, 35).

Characterization of the HIV-2 virion showed a similar genomic organization to HIV-1, even though they only share 40% to 60% of nucleotide homology (36). Interestingly, HIV-2 is genetically closer to the Simian Immunodeficiency Virus (SIV), with over 75% nucleotide homology (37), a result of interspecies transmissions from the natural host sooty mangabey (*Cercocebus atys*) as illustrated in *Figure 2* (27, 35, 38, 39).

SIV infects several nonhuman primates without causing disease or AIDS; such is the case of sootey mangabeys or african green monkeys, considered natural hosts for SIVsm or SIVagm, respectively (40-42). On the other hand, SIVsm can infect several species of Asian macaques, such as the rhesus macaque, causing severe immunodeficiency and AIDS–like syndrome (43, 44). Thus, the study of SIV infected macaques and comparative analyses of pathogenic and non pathogenic SIV infections have constituted important models for the study of HIV pathogenesis.

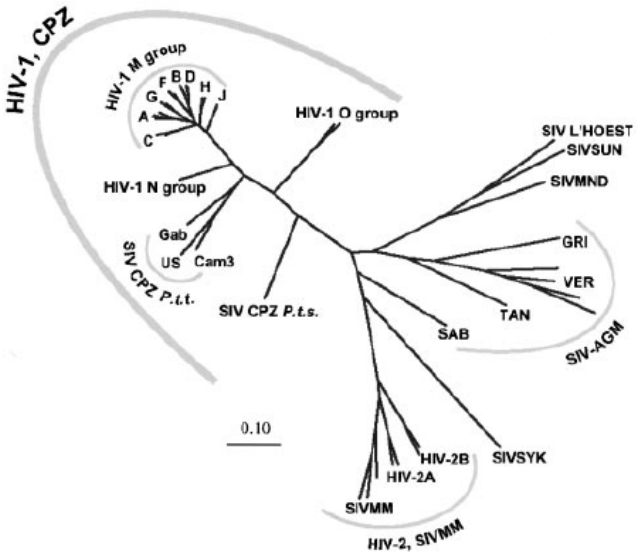


Figure 2 – Phylogenetic relationship of primate lentiviruses based on identity of *pol* gene sequences.

SIV_{CPZ} corresponds to SIV from chimpanzee (*Pan troglodytes*); SIV $_{L'HOEST}$ from L'Hoest's Monkey (*Cercopithecus lhoesti*); SIV_{SUN} from Sun-tailed Monkey (*Cercopithecus solatus*); SIV_{MND} from Mandrill (*Mandrillus sphinx*); SIV_{AGM} African Green Monkeys; SIV_{SYK} from Sykes' Monkey (*Cercopithecus albogularis*); SIV_{MM} from Sooty Mangabey (*Cercocebus atys*). Adapted from Reeves, *et al* (39).

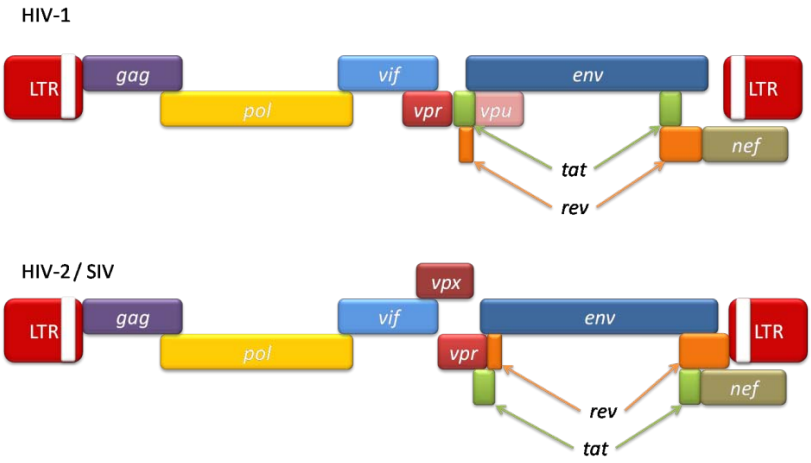


Figure 3 – Representation of the genomic organization of HIV-1 and HIV-2/SIV.

Adapted from Marlink, R. (27).

Replication cycle and latency

HIV-1, HIV-2 and SIV are members of the lentivirus genus included in the Retroviridae family, all characterized by the unique enzyme, reverse transcriptase which utilizes the viral genomic RNA as a template for synthesis of proviral cDNA (45).

The HIV genome is approximately 10kb, consisting of nine genes flanked by long terminal repeat sequences (LTRs) (46), depicted in *Figure 3*. The HIV-1's genome, like HIV-2 and all other retroviruses, has three major coding regions: *gag* which encodes for the structural proteins of the viral capsid, *pol* encoding enzymes involved in both viral replication and integration and *env* which encode the viral envelope glycoproteins. The genes *gag* and *pol* are well conserved in HIV-1, HIV-2, and SIV, and account for most of the cross-reactivity seen in enzyme-linked immunosorbent assays (ELISA) for HIV-1 (47, 48). HIV-1 and HIV-2 *env* genes encode for different glycoproteins (gp) that seem to have similar functions during infection; the outer membrane gp120 or gp105 and the transmembrane gp41 or gp36, respectively in HIV-1 or HIV-2. The LTRs are required for the integration of the viral cDNA (provirus) into the host cell genome and subsequent initiation of transcription of the integrated provirus (49). HIV-1 and HIV-2 share five additional reading frames *tat*, *rev, nef, vif* and *vpr*, coding for the gene products Tat, Rev, Nef, Vif and Vpr, accessory proteins with different key regulatory roles during HIV infection. HIV-1 *vpu* and HIV-2 *vpx*, although similar in sequence encode for proteins, Vpu and Vpx, respectively, that appear to have different functions in the infected cell (48). Most of the viral genes have overlapping reading frames, which allow the virus to encode several proteins in a small genome, through alternative splicing (50).

The virion capsid has a particular cone shape, contained within an external lipid envelope. Embedded in this membrane are the transmembrane glycoproteins responsible for binding to the host cells, and for fusion of the viral particle with the cell membrane, as illustrated in *Figure 4A*. Two copies of genomic RNA and several viral proteins such as reverse transcriptases, proteases and integrases are packaged inside the viral capside (51).

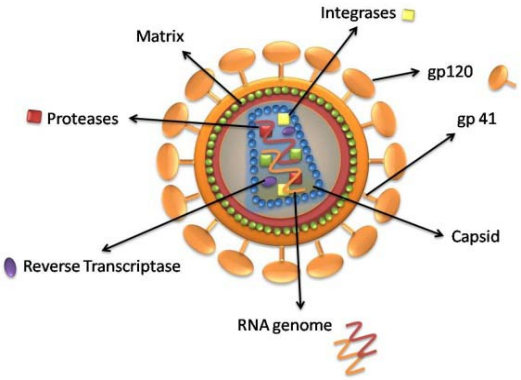
The viral replication cell cycle is schematically represented in *Figure 4B.* Both HIV-1 and HIV-2 enter cells by a membrane fusion process that requires the interaction of their external envelope gp with the CD4 molecule and co-receptors from the chemokine receptor family

(mainly CCR5 or CXCR4). The first step in cellular infection involves high affinity binding of the outer membrane gp to the CD4 molecule of host cells (52). Then, the outer membrane gp undergoes a conformational change allowing a better binding to either one of the cellular co-receptors (53). The induced conformational shift exposes a transmembrane gp which mediates cell-virus fusion. Its fusion domain binds and penetrates the host cell membrane bringing the two membranes closer (54), allowing virion content to enter the cytoplasm (55). The limited exposure of the key surfaces that mediate virus-host interaction during the extracellular stages of the HIV infection is considered a main reason for the failure to generate neutralizing antibodies that can control this phase of infection (56, 57).

Once in the cytoplasm, the RNA genome is reverse transcribed into double-stranded DNA by the viral reverse transcriptase. This process is highly error prone, leading to mutations of the viral genome (49). During the process of trafficking and transport of the viral DNA to the nucleus, the double stranded DNA and all viral proteins in the cytoplasm of the host cell aggregate, forming preintegration complexes (PICs). At this stage, PICs recruit host cellular factors to aid in the cell trafficking to the nucleus (58).

In the nucleus, integration occurs through the interaction of the viral integrase and of host cell DNA repair enzymes (59). Furthermore, the sites of HIV integration are not randomly distributed in the genome, but occur preferentially in transcriptionally active regions, downstream of promoters (60). When the provirus first starts to be transcribed, the messenger RNA is fully spliced leading to the production of Tat, Rev and Nef. Tat ensures transcription of viral mRNA (59). With the accumulation of Rev in the nucleus mRNA is exported leading to the production of the other viral proteins – Env, Vif, Vpr and Vpu or Vpx – and later in infection, of Gag and Pol (61). In the cytoplasm, the proteins are assembled to produce the viral capsid and envelope. The unspliced mRNA is packaged in the viral particles as well as the viral enzymes reverse transcriptase, integrase and protease (62). The completely assembled viral particles bud out of the cells, ready to infect other cells. All these processes critically depend upon the state of cell activation and cell cycling.

(A)



(B)

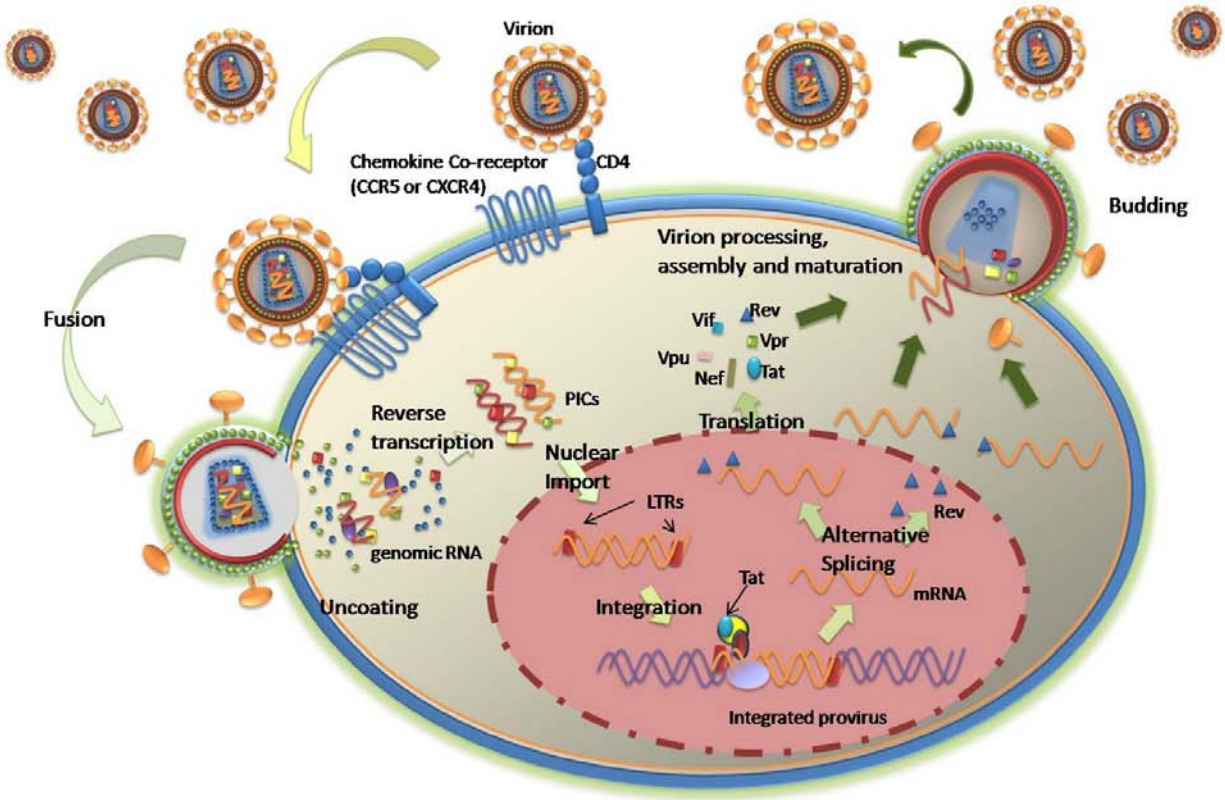


Figure 4 – The life cycle of HIV-1.

(A) Organization of the HIV-1 virion. **(B)** Schematic description of the events occurring after HIV infection of a susceptible target cell.

A state of latency is reached when integrated provirus have no active transcription. With the development of effective therapies that reduce the plasma viral load by targeting cells that are actively replicating virus, the persistence of a latent viral reservoir is considered the most important factor precluding the discontinuation of antiretroviral therapy (63-65). The factors that determine whether a cell will be latently infected or actively transcribing virus remain elusive.

Cell targets: co-receptor usage and tropism

The primary receptor for the HIV-1 particle is the CD4 molecule and, therefore, its main targets are CD4 T cells (66) and cells of the monocyte-macrophage lineage (67). Other cells have been shown to be infected *in vitro* though the *in vivo* relevance is not clear (68). HIV-2 infection of target cells has also been shown to require CD4 as the primary receptor together with one of several chemokine receptors as a co-receptor (69). HIV-2 appears to be more flexible in co-receptor usage than HIV-1, that mainly uses CCR5 or CXCR4 (53, 70 , 71, 72). Moreover, several HIV-2 strains have been shown to infect certain CD4-negative cell lines, generally through the use of CXCR4 or CCR5 alone (73, 74). However, in spite of the promiscuity of co-receptor usage exhibited by HIV-2 *in vitro*, several lines of evidence suggest that CCR5 and CXCR4 are as the major co-receptors for HIV-2 infection *in vivo* (75-77).

The chemokine receptors, CCR5 and CXCR4, belong to the family of transmembrane chemokine receptors.
CCR5 is upregulated on the surface of effector/memory T cells upon T cell activation and is a receptor for CCL3 (macrophage inflammatory protein 1α: MIP-1α), CCL4 (MIP-1β) and CCL5 (regulated upon activation, normal T cell expressed and secreted: RANTES). Interactions between these molecules regulate homing of effector/memory cells to specific lymphoid tissue (78). The importance of CCR5 as an HIV co-receptor, particularly during HIV-1 acute infection, became apparent when it was discovered that individuals homozygous for a mutation in the CCR5 gene (CCR5Δ32) were protected from HIV infection (79). CXCR4, the receptor for CXCL12 (stromal derived factor 1: SDF-1), is constitutively expressed on CD4 T cells, including naive T cells, and several other cell populations (78). These chemokines play diverse roles both during normal lymphocyte development and

during inflammation, varying from chemoattractant factors for monocytes and T cells, to activators of granulocytes or in homing of hematopoietic stem cell.

Tropism of the HIV particle is currently classified according to its binding to CCR5 (R5-Tropic) or CXCR4 (X4-Tropic). The R5 variants are associated with the primary infection, since they are the predominant phenotype in newly infected individuals. In around 50% of the HIV-1 infected patients in advanced disease stage, the viral phenotype switches to an X4 variant and this change is usually followed by a rapid decline in CD4 T cell numbers and progression to AIDS (80). An evolution from CCR5 to CXCR4 co-receptor usage is less clear in HIV-2-infected individuals. Many primary isolates have been shown to use a range of co-receptors including both CCR5 and CXCR4 and only a limited number of X4 viruses have been isolated from symptomatic patients (76, 81). In addition, it is not known whether primary infection is mainly associated with R5 HIV-2 strains as it has been described in HIV-1 infection (81, 82).

Natural history of HIV-1 infection

HIV-1 is transmitted by contact with infected body fluids, mainly by sexual contact, blood transfusion and/or contact with blood products, and by vertical transmission (from mother to child during pregnancy, delivery or breast feeding). Despite the multiple modes of transmission, there are no obvious differences in disease manifestations in individuals infected by different routes. This suggests that even if the initial target cells or route of HIV-1 infection differ, the subsequent viral spread ultimately results in a similar outcome (83).

Typical course of untreated HIV-1 infection

The acute phase of HIV-1 infection is characterized by viral dissemination throughout the lymphoid tissue. The highest levels of viremia are typically achieved during the acute infection. A selective depletion of memory CD4 T cells occurs coincidently with the active viral replication. Following exposure, around 50% of individuals experience an acute phase syndrome that may persist for up to several weeks, with symptoms ranging from a mild cold to a flu-like or mononucleosis-like disease. At the end of the acute stage, viremia decreases, mainly as a result of the expansion of HIV-1 specific CD8 T cells (84), but also due to the response of other components of the immune system, such as specific antibodies. Concomitantly, the CD4 T cell counts increase, although to values usually below the pre-infection levels. The parallel expansion of CD8 T cells, that is only partially due to HIV specific responses, causes an inversion of the CD4:CD8 ratio, which persists throughout the infection. The viral set point, attained through a balance between the viral replication and the host immune response, is predictive of the rate of progression to AIDS in untreated individuals (85), such that the higher the viral set point, the faster the disease progresses (86).

Recent studies have provided evidence for the role of the infection of gut-associated lymphoid tissue (GALT) in the profound CD4 depletion during acute infection. In the gut of acutely HIV-1-infected individuals a massive mucosal CD4 T cell depletion is observed, 4–6 weeks post-infection, much more marked than the decrease in CD4 T cell counts in the peripheral blood (87). It is important to note that the majority of CD4 memory T cells in uninfected humans resides in the GALT (more than 95%), with the remaining cells distributed by the blood (less than 2%), spleen, peripheral lymph nodes and other lymphoid tissues. As a consequence, CD4 memory T cell depletion is more rapid and profound in the

GALT during HIV-1 primary infection (88). Although this depletion is observed both in pathogenic and non pathogenic SIV infection (89, 90), the extent of this initial depletion has been suggested to determine the outcome of the HIV-1 infection.

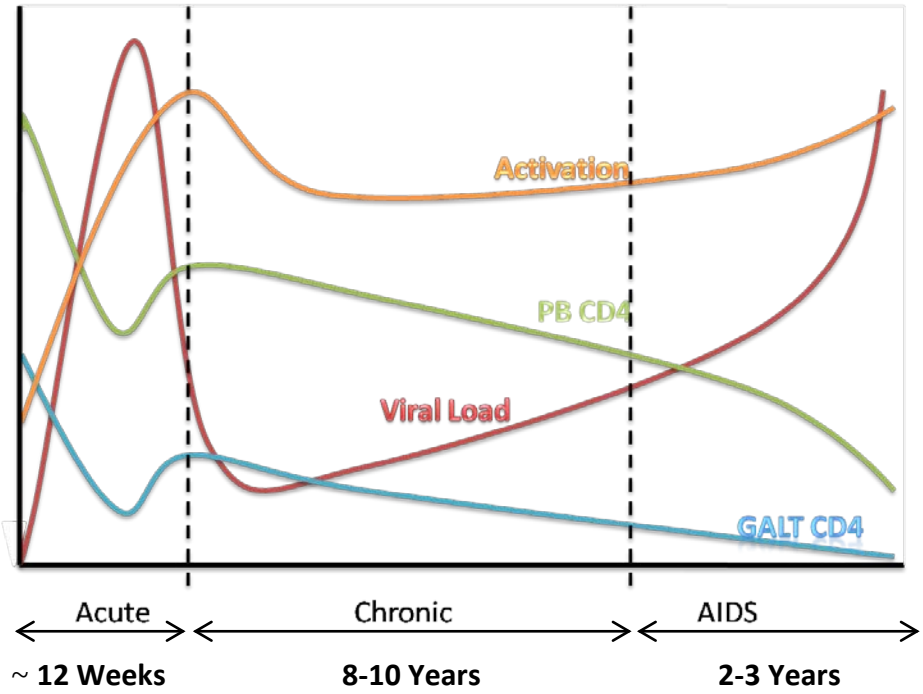


Figure 5 – Typical course of untreated HIV-1 infection.

Following resolution of the acute stage, a chronic persistent infection is established, that is associated with an asymptomatic period of clinical latency, prior to advanced immunodeficiency or AIDS. Numerous defects in immune function are evident in this phase of HIV-1 disease prior to substantial CD4 T cell loss (86). Despite the lack of clinical symptoms, ongoing virus replication can be detected consistently in lymphoid tissue attested by the usually significant levels of viremia. *In vitro* tests have shown that cell-mediated immune responses are progressively lost, first to microbial recall antigens, then to the more potent stimulation by alloantigens, and finally to mitogens. These *in vitro* functional T cell defects are also reflected *in vivo* by the loss of delayed type hypersensitivity skin testing (91).

Thus, despite a potent immune response and the marked downregulation of virus replication during the acute phase, HIV-1 succeeds in establishing a state of chronic infection with a variable degree of persistent viral replication.

The final stage of disease progression is characterized by an increased risk of opportunistic infections and tumors – AIDS, leading to death in 2 to 3 years in the absence of antiretroviral therapy (ART). Complex interactions between variable host responses and a highly variable pathogen determine the rate of the deterioration of the immune system. The median period from infection to AIDS is estimated to be 8-10 years, but AIDS can occur in months in the so called rapid progressors, whereas other infected individuals have remained asymptomatic for more than 10 years with relatively well preserved CD4 T cell counts, which are named long term non-progressors (91). A number of severe clinical manifestations, associated with reduced cell-mediated immunity in HIV-1 infected patients, are included in the Centers for Disease Control and Prevention (CDC) definition of AIDS. The AIDS-defining criteria also include a low CD4 T cell count (less than 200 CD4 T cells/μL blood). Since progression of HIV-1 disease is usually directly associated with a decrease in CD4 T cell counts and an increase in plasma HIV-1 viral load, measurement of these surrogate markers of infection has been used as a tool for assessing disease stage, prognosis and monitoring therapeutic response (86).

Antiretroviral therapy

Antiretroviral therapy or ART, using combinations of three or more drugs, is also termed highly active antiretroviral therapy in agreement with its major impact on slowing the progression of disease in a substantial proportion of adequately treated patients, by controlling viremia and allowing CD4 T cell counts to rise. It is the most important achievement in the battle against HIV-1 infection so far, with impressive impact on morbidity and mortality (92). Recent data from a comparative study of mortality before and after the introduction of ART in HIV-1 cohorts in Norway show that in the ART era the mortality was reduced by 80% (93). In another study, Walensky *et al* (94) demonstrated that since 1989, at least 3 million years of life have been saved in the United States as a direct result of ART.

Reservoirs

The number of available antiretroviral drugs has increased rapidly in the last decade, allowing for the use of several effective combinations of 3 or more drugs. However, strong evidence of the inability of ART to eradicate HIV-1 infection has come from studies of individuals who began antiretroviral therapy during the chronic stage of HIV-1 infection, showing that interruption of effective long-term antiretroviral therapy resulted in a rapid rebound of plasma viremia in almost all individuals (95, 96). Moreover, individuals whose ART was interrupted and remained off therapy for 4 to 6 weeks, experienced increases in the plasma HIV-1 RNA, to the same levels reached prior to the initiation of ART (97). These data suggest that ART does not eradicate HIV-1 infection and has no effect upon the post-therapy replication kinetics of the virus. HIV-1 proviral DNA and mRNA can be detected in CD4 T cells in individuals who have maintained "undetectable" plasma viremia (<50 copies of HIV-1 RNA/ml) for prolonged periods of time while receiving ART. Thus, viral reservoirs and some ongoing HIV-1 replication persist in the presence of effective antiretroviral therapy and it is currently consensual that complementary immune-based therapies will be required to eradicate or control the viral reservoirs.

HIV Immunopathogenesis

The hallmark of HIV infection is the progressive CD4 T cell depletion associated with active viral replication. There is considerable controversy regarding the relative contribution of the various putative mechanisms to the depletion of CD4 T cells during the course of HIV infection.

Tap and drain hypothesis

In 1995, Ho and colleagues (98) proposed that the virus was the major driving force behind the CD4 depletion, whether it was by direct infection and concomitant cytolytic effects, or by the removal of the infected cells by the immune response. This was termed the "tap and drain" hypothesis. In this analogy a sink represented the CD4 T cell compartment, an unplugged drain depicted the impact of HIV infection on CD4 numbers and water flowing from an open tap symbolized the production of new CD4 T cells. Over time the open tap, i.e., the production of new CD4 T cells would be unable to counteract the "flow of water going down the drain", ultimately resulting in the depletion of the CD4 compartment. The major evidence that supported this hypothesis came from the study of the reduction of viremia and increase in CD4 T cells in the blood after treatment with a protease inhibitor (98, 99). Mathematical modeling applied to these data estimated that everyday there was a destruction of $2x10^9$ lymphocytes by the virus, which was compensated by the regenerative capacity of the immune system in the steady state (98-100). In this model, the ability to produce new cells progressively deteriorates resulting in AIDS. This mathematical model predicted that the half-life of the infected CD4 T cells was such that in 10 years the CD4 compartment would be empty and also that continuous therapy use would be able to eradicate infection in less than 2 years (98-100).

One of the first observations that directly challenged this simplistic view was that the initial rise in CD4 T cells did not signify the production of new cells as predicted. In fact, the increase in CD4 T cell counts that occurred immediately after initiation of therapy was subsequently shown to result mainly from the redistribution of CD4 T cells into the blood stream, that were trapped in the lymph nodes and other tissues, due to decrease in antigen load (101, 102). Also, the frequency of infected CD4 T cells was not sufficient to explain the CD4 T cell depletion by direct virus killing (103). Moreover, the major CD4 depletion could

not be explained in such a way, as the virus preferentially infects activated T cells and these cells are expected to die as a result of activation induced cell death (AICD) during the contraction of the immune response, as stressed by Grossman *et al* (104-106).

Chronic hyper immune activation

Studies on non pathogenic SIV infection, such as SIV infection of sootey mangabeys (SIVsm), showed that, despite the presence of very high viral load, there was no chronic depletion of CD4 T cells (107). On the other hand, when a susceptible species like rhesus monkeys were infected with SIVsm, there was a major CD4 depletion that correlated with viral load. This depletion was also correlated with an upregulation in activation markers, not found in SIV infected sootey mangabeys, where only a transient or no upregulation of activation markers was found (108, 109).

These observations support the proposition of a new paradigm of HIV pathogenesis, where the driving force behind CD4 depletion is chronic hyper-immune activation. Several other lines of evidence sustain a major role of chronic activation on AIDS progression. Liu *et al* (110) showed that increased expression of the activation marker CD38 on T cells positively correlated with poor disease prognosis. Additionally, a high level of apoptosis associated with bystander activation was documented in non-infected cells including CD8 T cells (111) that was shown to contribute to the disruption of the immune system. This chronic activation was characterized by increased, sustained frequencies of lymphocytes expressing activation markers, not only CD38, but also others like HLA-DR, and an increased frequency of cycling cells as assessed by Ki-67 (112, 113). In fact some of the first markers of disease progression to be used, before viral quantification, were β2-microglobulin and neopterin, which are markers indicative of immune activation. Moreover, data from our laboratory further support a role of immune activation in AIDS pathogenesis. Sousa *et al* (112) showed that CD4 depletion is directly linked to immune activation both in HIV-2 and HIV-1 infection and only indirectly to viral load (106, 112).

Chronic hyper activation has a major impact on immune function through many mechanisms: inducing functional cellular impairment; damaging the local lymphoid tissue architecture; altering cell trafficking; promoting selective depletion of resting naive and memory CD4 and

CD8 T cells. Moreover, even though quiescent CD4 T cells can be infected with HIV, reverse transcription, integration, and virus spread are much more efficient in activated cells (114). In addition, it has been demonstrated that cellular activation induces expression of virus in latently infected CD4 T cells (115).

An increase in microbial translocation associated with the perturbations of the gut mucosa documented in HIV and SIV infections as been suggested as one of the possible contributing factors to the pan-immune activation (87, 116). The increase of bacterial products such as LPS in the blood would contribute to maintain monocyte/macrophage, dendritic cells and B cells in a continuous state of activation.

Currently, HIV disease progression is thought to result from a multifactorial process, mainly driven by persistent immune activation induced by antigens and inflammatory factors, modulated both by viral determinants and host genetics factors.

Ability to counteract CD4 depletion

Regardless of the mechanisms involved in the CD4 depletion, the ability to replenish the T cell pool is a major determinant of disease progression.
HIV-associated chronic immune activation results in constant recruitment of CD4 T cells from the naive into the memory-effector activated pool. This creates a constant pressure on the replenishment mechanisms of the CD4 populations. Maintenance of the naive T cell pool is currently thought to be due to mechanisms of homeostatic control of post-thymic T cells as well as of an age-dependent contribution of thymic emigrants (117). Thus direct or indirect impact of HIV on either *de novo* T cell production, or thymus independent homeostatic mechanisms, can further influence the course of HIV infection. The ability to counteract CD4 depletion during HIV-1 and HIV-2 infection represent the focus of this work.

T cell production in HIV disease: relative contributions of thymic output and peripheral T cell homeostasis

Peripheral T cell proliferation and IL-7 as a critical homeostatic cytokine

Homeostasis is a state of equilibrium attained when a given population has similar gains and losses of its components. When in a steady state, the peripheral pools of naive and memory T cell populations occupy their space in the lymph nodes and all other sub-compartments of the immune system, circulating between them, occasionally dividing or dying.

Homeostatic mechanisms and their modulators may be different for different sub-populations of lymphocytes. Both the CD4 and CD8 naive and memory T cell pools are confined to different locations; require different stimuli to survive and to perform their function; and likely obey different "sets of rules" for the maintenance of their numbers. Although these rules remain largely unclear, in steady state conditions, memory T cells are thought to compete for survival signals with other memory T cells but not with naive T cells (118, 119), thus occupying different "niches" (120). Increasing evidence suggests that the homeostasis in these different niches is regulated through competition for limiting resources. Naive T cells have been shown to require continuous contact with self-peptide bound to the major histocompatibility complex (MHC) molecule and/or cytokines such as interleukin (IL)-7. Memory CD8 T cell survival is controlled mostly by IL-7 that provides a survival signal and IL-15 that is thought to mediate proliferation (121). The factors that control memory CD4 T cells turnover and survival are less clear. Although a significant part of these studies have been performed in mouse models, it is thought that similar mechanisms apply to human's T cell homeostasis.

IL-7 is considered a key cytokine in T cell homeostasis acting both during thymopoiesis and in the periphery, where it promotes T cell proliferation and survival, particularly of the naive pool (122-124). IL-7 is constitutively produced by stromal cells of the bone marrow, thymus, mucosal lymphoid tissues, and lymph nodes (125-128).

The potential of IL-7 to restore immune competence through the combined effects of increased thymic output (129) and enhanced peripheral homeostatic expansion of T cells (130, 131), highlights this cytokine as a potent modulator of T cell immune reconstitution

(132). The IL-7 receptor is composed of a α chain and a common γ chain, shared with a family of cytokines, namely IL-2, -4, -9, -15 and -21. The expression of the IL-7Rα (CD127) has a critical role in the regulation of the IL-7 biology (133, 134). IL-7 signaling results in a transient down-regulation of the IL-7Rα that is thought to allow adequate sharing of available IL-7 by a large number of T cells (135).

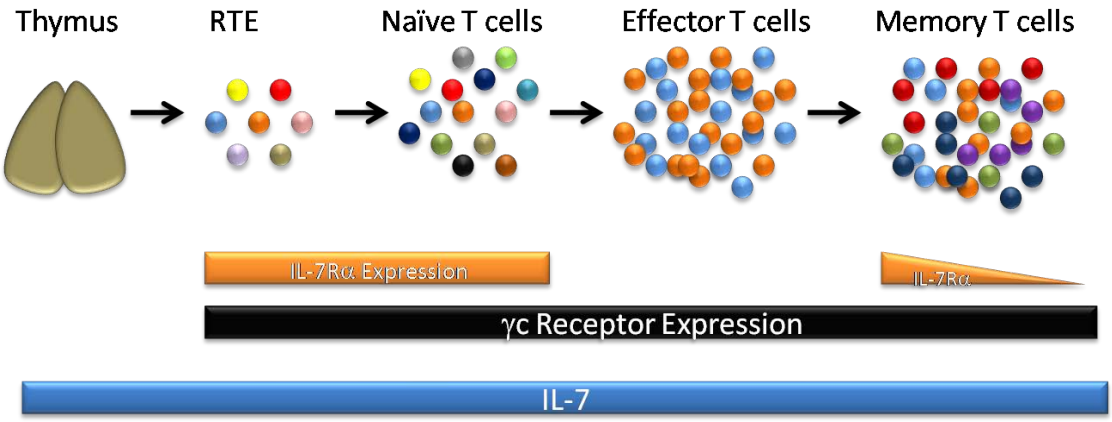


Figure 6 – Schematic representation of changes in IL-7Rα expression during post-thymic T cell development.

Recent thymic emigrants (RTE) display high levels of IL-7Rα and are highly responsive to IL-7. Although the expression of IL-7Rα is diminished transiently following the activation of T cells, both CD4 and CD8 naive and memory T cells express IL-7Rα. Adapted from Snyder, *et al* (136).

In lymphopenic states, such as those found following radiotherapy or chemotherapy, or in some acute viral infections, such as measles, homeostatic mechanisms ensure the replenishment of T numbers (137-139). Thus these homeostatic mechanisms play a pivotal role in assuring that the depleted T cells populations are replaced.

Circulating IL-7 levels increase in lymphopenic states, either due to increased production resulting from a compensatory feedback loop, or increased availability due to the reduction of cell targets (128, 131). Furthermore, clinical studies have reported an increase in circulating levels of IL-7 in the settings of HIV-1 induced T cell depletion, idiopathic CD4 lymphopenia and following cancer chemotherapy, which returned to normal levels upon recovery of the T

cell populations (128, 131). Thus, the clarification of the role of the IL-7 network in the maintenance of the T cell pool is of major importance.

T cell production in the thymus

The thymus is the central lymphoid organ where T lymphocytes develop and mature. It is therefore essential in the establishment of the peripheral T cell pool and the in the generation of a diverse T cell receptor (TCR) repertoire. TCR gene rearrangement plays a pivotal role in thymocyte assembly of a functional TCR and at the same time ensures that T cells have different specificities. In order for this to occur, receptor assembly proceeds in stages, each stage being verified for correct assembly. Developing thymocytes undergo a series of distinct phases, marked by changes in the rearrangement status of their TCR genes that are associated with varied levels of expression of cell surface proteins, such as TCR, CD3 complex, CD4 and CD8. These surface changes reflect the state of functional maturation of the cell. Thus, particular combinations of cell-surface proteins can be used as markers for thymocytes at different stages of differentiation. Early in T cell development, cells commit to two main distinct lineages: αβ and γδ. Later, αβ T cells develop into distinct functional subsets: CD4 and CD8 T cells (140).

The earliest lymphoid progenitors, termed common lymphoid precursors (CLP) are derived from bone marrow hematopoietic stem cells. Interactions with the thymic stroma trigger an initial phase of differentiation along the T cell lineage pathway followed by cell proliferation and the expression of the first cell-surface molecules specific for T cells. At this stage the thymocytes bear distinct markers of T cell lineage, but still lack the CD3-TCR complex and CD4 or CD8, being termed double-negative (DN) thymocytes.
During the DN phase, rearrangement of the TCR genes occurs. The genes that encode the variable regions of the TCR α, β, γ, and δ chains are assembled through recombination of their multiple variable (V), and joining (J) gene segments. In addition the TCRβ and TCRδ loci have diversity (D) segments as well as V and J segments to be rearranged. The TCRδ locus is embedded within the TCRα locus in such a way that any V-Jα rearrangement automatically deletes the TCRδ locus entirely (140). The particular pathway that the cells follow depends on whether they successfully rearrange D-Jβ and V-D-Jβ, generating a productive TCRβ open-reading frame before they have completed productive rearrangements

of both the γ and δ loci. In the first case, they develop into αβ T cells; in the second case, they develop into γδ T cells.

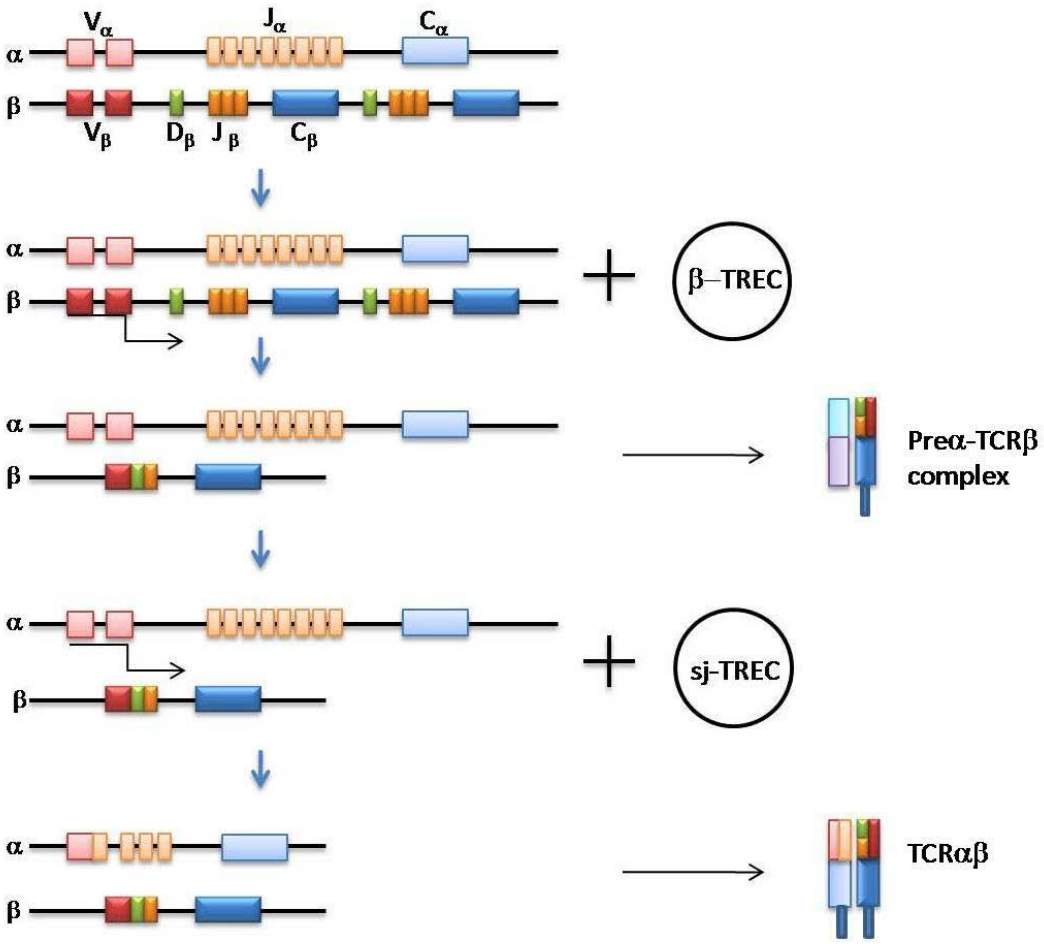


Figure 7 - TCR α and β chains gene rearrangement.

Representation of the rearrangement of the TCR α and β chains and also of the episomal DNA circles generated during excisional rearrangement of TCR genes, T cell receptor excision circles (TREC). Adapted from *Paul et al* (140) and Janeway *et al* (141).

The rearranged TCRβ then dimerises with a pre-T-α chain, forming the pre-TCR that is co-expressed on the cell surface. Productive assembly results in the arrest of further β rearrangement, leading to cell proliferation and subsequently expression of CD4 and CD8 that defines the double-positive (DP) stage.

The TCRα rearrangements occur mostly during the DP stage, following multiple rounds of proliferation. A complete TCR is then presented on the surface of the DP cell, complexed with the CD3 molecules. Thymocytes are subsequently tested for TCR functionality and self-MHC interaction, under extremely stringent conditions, with only a small proportion of cells surviving. These processes are necessary to determine whether the newly expressed TCRαβ has sufficient affinity for MHC molecules to survive. On the other hand, thymocytes that have strong interactions with self-antigens in the thymus are deleted in order to prevent autoimmunity. Also during this process the TCR-MHC interactions, guide or select cells to develop into the two main αβ T cell subsets. Cells with a TCR that recognize MHC class II molecules develop as $CD4^+$ cells, whereas those with TCR that recognize MHC class I molecules develop as $CD8^+$ cells by mechanisms that are not yet fully clarified. Single positive CD4 or CD8 αβ T cells leave the thymus and are incorporated into the peripheral naive T cell pool (140).

Direct HIV impairment of the thymus

Evidence that suggests that HIV can impair or reduce thymic function has been accumulating. Thymic structure assessed in postmortem analysis of AIDS infected patients shows severe destruction of architecture and reduced thymocyte numbers. HIV-1 infection of the thymus *in vivo* has been confirmed (142-144) as a consequence of the thymocyte expression of the primary HIV-1 co-receptors, CXCR4 and CCR5. It has been shown that both R5 and X4 tropic viruses can enter and complete reverse transcription in most thymocyte subsets (145, 146). Also, Sopper *et al* (147) showed that CD4 thymocytes decrease during chronic stage of SIVsm infection in the rhesus macaque model. However, in a small clinical study on HIV-1 patients that had been thymectomized prior to infection, it was shown that there was no significant alteration in disease progression in comparison to the non-thymectomized HIV-1 infected control cohort (148). Also in juvenile rhesus macaques thymectomies had apparently little impact on naive T cell production (149). Given these data, the impact of a functional thymus on HIV progression is an important subject to study. The ability to assess thymic

function or lack thereof, which may be evaluated by measuring thymic output (i.e. the number of T cells exiting the thymus per day), is essential to understanding the impact of alterations to this process on the correct functioning of the immune system during a persistent chronic infection like HIV. For that purpose the identification of markers that define recent thymic emigrants (RTE) is of major importance and substantial technical efforts have been devoted to identifying such a phenotype.

Markers of thymic function upon HIV infection

Indirect methods have been used to evaluate thymic function, which include determination of naive T cell subsets and naive/memory imbalances. The simultaneous expression of CD45RA and CD62L on the surface of CD4 T cells is currently thought to identify the majority of the naive population, while for CD8 T cells the simultaneous surface expression of CCR7, CD45RA and CD27 is considered a naive phenotype (112). However, in the setting of HIV infection, the persistently increased flow of T cells from the naive to the memory compartment makes these analyses difficult.

Computer tomography scans of the chest have been used to correlate the thymic sizes of HIV-1 infected individuals with their CD4 naive ($CD45RA^{+}CD62L^{+}$) lymphocyte counts, but such an imaging technique cannot reliably measure the true lymphoid mass of the thymus because of the difficulty to accurately discriminate thymic from adipose tissue (150).

The detection of the episomal DNA circles generated during excisional rearrangement of TCR genes, was postulated to be a reliable measurement for RTE status. These episomal DNA circles, termed T cell receptor excision circles (TREC) are the “by-products” of TCR gene rearrangement events that occur during thymopoiesis (depicted in *Figure 7B*). (151-156). Since TREC are not regenerated upon subsequent division of TREC-containing cells, their numbers are diluted-out upon cellular proliferation (155-158). Thus, the detection and quantification of TREC in peripheral T cells serves as a marker for the recent thymic emigrant population (152). In this case though, “recent” denotes naive T cells that have undergone no more than a few cellular divisions since leaving the site of TCR rearrangement and does not necessarily reflect the time elapsed since the rearrangement event (159).

As the TCRα chain loci are the last to undergo rearrangement, the excised DNA containing the δ loci, termed sjTREC, was primarily used by Douek *et al* (155) as a marker for RTE to study thymic function in HIV infected patients. As expected, in a group of healthy controls, decreasing numbers of sjTREC were observed with aging reflecting a gradual loss of thymic function, although it was maintained until old age. Interestingly, it was also reported that sjTREC were reduced in HIV-1 infected individuals and increased following treatment with ART, which suggested impairment of thymic function during HIV-1 infection. However, in view of the consequences of the generalized chronic immune activation reported during HIV-1 infection, the rapid decline in sjTREC could simply be due to changes in peripheral T cell division rates. Thus, TREC may be lost by increased cell division in the periphery and not by a diminished thymic output (157, 160).

Importantly, Dion *et al* (161) proposed a marker for measuring thymic function, based on quantification of TREC molecules generated during both TCRβ and TCRα chain loci rearrangement events. Thus, the βTREC, resultant from the TCRβ gene rearrangements, are quantified in addition to the sjTREC (156) and then a sj/β TREC ratio is calculated (162, 163). This ratio is independent of peripheral T cell proliferation and death rate in the periphery. Thus, the sj/β TREC ratio directly reflects the number of proliferative cycles undergone by precursor T cells during their intrathymic differentiation, which has been shown to directly correlate with thymic output. Therefore, the quantification of the βTREC, the sjTREC and the sj/β TREC ratio provides a more accurate measure of thymic function, as illustrated in *Figure 8* (161). Results generated using this technique show suppression of intrathymic proliferation in HIV-1 infected patients in the initial phase of disease (161). Moreover, it was recently demonstrated the slow progression of HIV-1 disease and the maintenance of circulating CD4 T cells are strongly associated with efficient thymopoiesis (162).

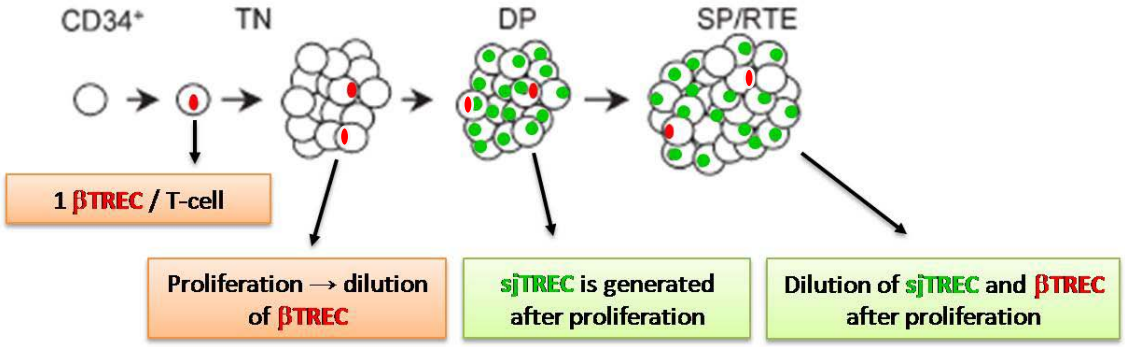


Figure 8 – Schematic representation of the effect of intrathymic proliferation on βTREC and sjTREC numbers.

TN: Triple Negative stage; DN: Double Positive stage; SP/RTE: Single Positive/ Recent Thymic Emigrant stage. Adapted from Dion, *et al* (161).

T cell surface markers that clearly identify an RTE subpopulation and can be quantified by techniques such as flow cytometry, exist for some species, such as chicken (chT1) (152) and rat (Thy-1, RT6) (164), but no equivalent marker has been identified in human T cells. Kimmig *et al* (165) proposed CD31 as a surface marker for cells that recently left the thymus, based on the observations that the subdivision of naive $CD45RA^+$ T cells according to CD31 expression has an impact on the amount of TREC quantified, since the $CD31^+CD45RA^+$ T cell subpopulation has an enrichment in TREC and the subset of naive $CD31^-$ $CD45RA^+$ T cells has lower TREC levels (165).

Each of these measurements has its advantages and disadvantages, therefore, combinations of these parameters can provide a picture of the contribution of thymic output and peripheral homeostatic expansion to maintenance of T cell populations.

Impact of HIV-1 on the maintenance of the CD4 T cell pool

In order to study the impact of HIV-1 on the mechanisms of the naive CD4 T cell pool replenishment, it is of major importance to clarify the role of the IL-7 network as a peripheral homeostatic factor and also to address the role of maintenance of thymic output in disease progression. The present work took advantage of the study of an attenuated model of HIV disease, the second human immunodeficiency virus, HIV-2.

Models to study HIV-1 infection

Understanding the host immune and viral factors that contribute to protection from infection or disease progression remains elusive, despite years of research on HIV-1.

A common strategy to investigate HIV immunopathogenesis has been to use natural models of HIV-1 infection with unusual clinical outcomes, such as the so called long-term non-progressors (LTNP). LTNP are usually considered to be those individuals who have been infected with HIV for a long period (e.g. more than10 years), with CD4 T cell counts in the normal range that remain stable over time, in the absence of ART, irrespective of their viremia (166). More recently, much effort has also been put into the study of the rare individuals that maintain plasma viral RNA at <50 copies per milliliter without ART. These "Elite controllers" are estimated to represent less than 0.1% of all HIV-1 infected patients. When these more stringent definitions based predominantly on levels of plasma viremia are applied, very strong genetic associations with HLA B*5701 and HLA B*2705 alleles have been found (167, 168). Despite this, these groups have been shown to be heterogeneous. Many different factors have been implicated such as variability in individuals' genetics, immune response to the virus and/or the possibility of infection with attenuated strains of HIV. Thus, the study of LTNP/Elite controllers is very restricted to the small number of individuals that can be adequately paired in each study cohort.

Other approaches to study the immunopathogenesis of HIV have been provided through the study of non-human primate models of infection (169) and numerous insights related to disease progression have come from studies of pathogenic and nonpathogenic SIV infections (170). In their natural hosts, SIV infections cause no disease, whereas SIV infection in susceptible monkeys causes severe immunodeficiency and an AIDS-like syndrome (171). Thus, these are two comparable models of infection with different outcomes, even though extrapolation of nonhuman primate models to human HIV has many limitations, for instance the different species' life-spans and the time of progression to AIDS (2 years vs 10 years on average, respectively).

There are no other animal models to address AIDS pathogenesis and although lots of expectation has surrounded the humanized mice model, there are still significant limitations in the investigation of T cell homeostasis (172).

HIV-2 as a model of attenuated disease

HIV-2 is an AIDS causing retrovirus apparently able to generate the same clinical spectrum as HIV-1. However, in HIV-2 infection viremia is much reduced, in most cases undetectable, and the rate of CD4 T cell depletion is much slower than in HIV-1 infected patients. Therefore, as initially stated, HIV-2 infection is considered an attenuated form of HIV disease and a very important model for the study of basic pathogenic mechanisms in HIV/AIDS and the identification of new targets for therapeutic interventions.

HIV-2 infected individuals have very low to undetectable viremia

As already mentioned, during the course of HIV-2 infection, the levels of virus in circulation are usually undetectable. At first, it was thought that the aviremia in individuals infected with HIV-2 was due to the use of viral quantification techniques that were developed for the quantification of HIV-1 viral load. But the development of PCR-based techniques to quantify the HIV-2 RNA copies in plasma confirmed that the level of viremia in all stages of infection is much lower than in HIV-1 and, that in most cases, it is below the limit of detection (25, 173), a finding that had been predicted by the reduced frequency of successful virus isolation from HIV-2 infected patients (174). Interestingly, the quantitative assessment of HIV-2 provirus load documented levels similar to those observed in HIV-1 infected individuals (175, 176). The levels of HIV-2 cell-associated proviral load suggest that the virus is able to disseminate and establish viral reservoirs as efficiently as HIV-1.

As a consequence of the very low to undetectable viral load, the likelihood of HIV-2 transmission is much lower than that for HIV-1 infection (20, 23). Reduced vertical transmission rates of 0 to 4% of HIV-2-positive pregnancies (26, 177, 178) which is quite distinct from the vertical transmission rates of 33% of in HIV-1-positive pregnancies, in the absence of antiviral therapy (178-180). Also, HIV-2 appears to be less readily transmitted by sexual contact (26) and the efficiency of heterosexual transmission of HIV-2 is estimated to be five times lower than that of HIV-1 (181, 182). Interestingly, the prevalence rates of HIV-2 infection increases among older men and women (23, 183, 184), suggesting that HIV-2 has been present in West Africa for several generations. The lower sexual transmission rate of HIV-2 compared with HIV-l may help explain the reason for the more confined geographical distribution of HIV-2, whereas HIV-l has spread worldwide (18).

Decline in CD4 T cell counts during progression of HIV-2 disease

In HIV-2 infected individuals, CD4 T cell counts decline progressively, as in HIV-1 infection, but the rate of CD4 T cell decline is slower with a longer asymptomatic period and a limited impact on the survival of the majority of infected adults (24, 185, 186). In a recent longitudinal study in French cohorts by Drylewicz *et al* (187), the rate of CD4 T cell decline was calculated for both infections. In HIV-1 infection, CD4 T cell counts declined at a rate of 49 cells/μl per yr, whereas in HIV-2 infection this rate was only 9 cells/μl per yr.
Despite this, it is very important to note that in cross-sectional studies of immunologic parameters it is possible to pair HIV-1 and HIV-2 patients at a similar disease stage with the same CD4 T cell counts, which is not possible in other clinical models of HIV pathogenesis, like LTNP. These types of comparisons allow the impact of CD4 depletion *per se*, on the parameters under evaluation to be taken into account.

Comparable chronic immune activation during HIV-2 and HIV-1 infections

As previously mentioned the pathogenic effects of HIV-1 have been linked to a state of severe immune activation and most likely are not solely dependent upon direct infection and viral killing. Thus it was hypothesized that one possible reason for the less detrimental effect of HIV-2 infection on the host could be due to a lack of generalized immune activation. However, Sousa *et al* in 2002 (112) reported that HIV-1 and HIV-2 patients with a similar degree of CD4 depletion displayed similar levels of T cell hyper-immune activation and similar numbers of cycling cells in the peripheral blood despite large differences in the plasma viral load. HIV-1 and HIV-2 infected patients were grouped according to levels of CD4 depletion and it was observed that with this categorization, the two infections exhibited a similar spectrum of immune perturbations: imbalances in the naive/memory-effector population ratios; comparable upregulation of CD4 and CD8 T cell activation markers (HLA-DR, CD38, CD69, Fas molecule); a similar increase in the frequency of cycling CD4 T cells ($Ki67^+$), which was in strong correlation with the expression of activation markers; and a similar level of anergy, as assessed by the *in vitro* proliferative responses to CD3 stimulation and to a panel of microbial antigens. Thus, for the same level of CD4 T cell depletion, HIV-1 and HIV-2 infected patients exhibited similar elevations in the frequencies of activated and cycling T cells.

Study of factors that determine CD4 T cell replenishment in HIV-2 infection

However, despite the same levels of activation at a given degree of CD4 depletion, HIV-2 patients are known to progress to AIDS more slowly, suggesting an increased ability to counteract the CD4 loss associated with immune activation. It is thus plausible that the mechanisms of CD4 T cell replenishment are better maintained in HIV-2 than in HIV-1 infection, and that this may be a decisive contributing factor to the slower rate of CD4 T cell depletion in HIV-2 infection.

The main advantage in using this clinically attenuated model of infection is that it is possible to study the differences in mechanisms of naive CD4 T cell pool replenishment in HIV-2 and HIV-1 infection, in cohorts of patients that are paired for age and disease stage. The final goal is to provide insights into the mechanisms of replenishment of the CD4 T cell pool, which is fundamental in understanding the pathogenesis of HIV infection.

Aim

The main objective of this work was to contribute to the understanding of the relative importance of factors involved in HIV/AIDS immunopathogenesis, specifically focused on those related to the replenishment of the naive CD4 T cell pool through peripheral homeostatic mechanisms and/or thymopoiesis. HIV-2 infection was used as a model of attenuated HIV disease. In comparison with HIV-1 infection, HIV-2 is characterized by a much slower progressive CD4 T cell decline, although with similar levels of hyperimmune activation for the same degree of CD4 depletion, despite the reduced viremia.

Firstly this work aimed to clarify the role of IL-7 network in the maintenance of the T cell pool by comparing HIV-1 and HIV-2 infections. IL-7 is a non-redundant cytokine for T cell homeostasis that acts in the periphery promoting proliferation and survival of naive and memory-effector T cell pools. HIV-1 infection is associated with an increase of circulating IL-7 levels, in direct correlation with the decrease in CD4 T cells, and is also associated with a decreased expression of IL-7Rα on T cells. Circulating levels of IL-7 were quantified in serum of HIV-2 and HIV-1 infected patients stratified according to CD4 T cell depletion. Also, longitudinal studies of circulating IL-7, in parallel with CD4 T cell counts, were performed in HIV-2 patients to further characterize the relationship between increasing IL-7 and decreasing CD4 T cell numbers. Quantification of IL-7Rα (CD127) was performed using flow cytometry in parallel with markers of naive and effector-memory CD4 and CD8 T cell subpopulations in HIV-2 and HIV-1 infected patients.

Secondly, the contribution of thymic output to disease progression rate was addressed. To study alterations in thymic function, Dion *et al* (161) recently developed methodologies that allow the quantification of excision circles resulting from different intrathymic TCR gene rearrangement (TREC), sj and βTREC and the calculation of a sj/β TREC ratio (162, 163). This marker, which is independent of peripheral T cell proliferation and death rate, directly reflects the number of proliferative cycles undergone by precursor T cells during their intrathymic differentiation, thereby providing a more accurate measure of thymic function (161). Thus, a collaboration with Dr. Cheynier's group was established to study sj and βTREC in PBMCs of HIV-2 and HIV-1 infected patients in order to evaluate sj/β TREC ratio relationship with the rate of CD4 T cell decline.

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Results

RATE OF INCREASE IN CIRCULATING IL-7 AND LOSS OF IL-7Rα EXPRESSION DIFFER IN HIV-1 AND HIV-2 INFECTIONS: TWO LYMPHOPENIC DISEASES WITH SIMILAR HYPERIMMUNE ACTIVATION BUT DISTINCT OUTCOMES.

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Rate of Increase in Circulating IL-7 and Loss of IL-7Rα Expression Differ in HIV-1 and HIV-2 Infections: Two Lymphopenic Diseases with Similar Hyper Immune-Activation but Distinct Outcomes.

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Abstract

IL-7 is a non-redundant cytokine for T cell homeostasis. Circulating IL-7 levels increase in lymphopenic clinical settings, including HIV-1 infection. HIV-2 infection is considered a "natural" model of attenuated HIV disease given its much slower rate of CD4 decline than HIV-1 and limited impact on the survival of the majority of infected adults. We compared untreated HIV-1 and HIV-2 infected patients and found that the HIV-2 cohort demonstrated a delayed increase in IL-7 levels during the progressive depletion of circulating CD4 T-cells as well as a dissociation between the acquisition of markers of T-cell effector differentiation and the loss of IL-7Rα expression. This comparison of two persistent infections associated with progressive CD4 depletion and immune-activation demonstrates that a better prognosis is not necessarily associated with higher levels of IL-7. Moreover, the delayed increase in IL-7 coupled with sustained expression of IL-7Rα suggests a maximization of available resources in HIV-2. The observation that increased IL-7 levels early in HIV-1 infection were unable to reduce the rate of CD4 loss and the impaired expression of the IL-7Rα irrespective of the state of cell differentiation raises concerns regarding the use of IL-7 therapy in HIV-1 infection.

Introduction

Interleukin-7 (IL-7) is considered a key cytokine in T-cell homeostasis acting both during thymopoiesis and in the periphery promoting naive T-cell proliferation and survival, as well as the maintenance of memory cells (1-4). Thus, much expectation has been placed on the therapeutic use of IL-7 to improve thymic output and broaden the T-cell repertoire in lymphopenic clinical settings, as well as an adjuvant to improve the breadth of vaccine responses (5-10). IL-7 is constitutively produced by stromal cells of the bone marrow, thymus, mucosal lymphoid tissues, and lymph nodes (11-14). Circulating IL-7 levels increase in lymphopenic states, suggesting either a feed-back compensatory increased production or an increased availability due to the reduction in cell targets (1, 14-16). Increasing data point to a critical role of the expression of the α-chain of the IL-7 receptor (IL-7Rα) in the regulation of IL-7 biology (17, 18). IL-7 signaling results in a transient down-regulation of the IL-7Rα that is thought to allow adequate sharing of available IL-7 by a large number of T-cells (17). Moreover, IL-7Rα can also be down-regulated by other cytokines that share the common cytokine receptor chain γc, such as IL-2, and by TCR stimulation (19, 20). Of note, differentiated effector T-cells have been shown to be IL-7Rα^{low}, a phenotype associated with decreased survival and proliferation in response to IL-7 (21, 22). HIV-1 infection is associated with high levels of circulating IL-7 (1, 14, 23-25), as well as with a decreased expression of IL-7Rα on T-cells (24-29).

HIV-2, the second AIDS virus, is considered a "natural" model of attenuated HIV disease because it is associated with a much slower course of disease progression than HIV-1 with limited impact on the survival of the majority of infected adults (30, 31). More than 90% of HIV-2 infected individuals are thought to meet the standard criteria for "long-term non-progressors" (30-32), but display a steady decline of CD4 counts (30, 32-34). Although the rate of CD4 depletion is markedly slower in HIV-2 than in HIV-1 infection, a direct correlation with the levels of immune activation was observed in both cases (34, 35). This is despite the low levels of viremia that characterize all the stages of HIV-2 disease (33, 34, 36-38). The reduced viremia is considered the main reason why HIV-2 infection remains confined to West Africa (39-41). In Portugal, due to its connections with its past-colonies, there is a significant prevalence of HIV-2, currently accounting for 5% of the HIV infections (42).

We took advantage of this situation and compared untreated HIV-1 and HIV-2 infected patients living in Portugal in order to obtain insights into the role of IL-7 in the rate of CD4 decline and the imbalances of T-cell subsets associated with HIV/AIDS pathogenesis as well as on the regulation of IL-7 in other lymphopenic states.

We found that a delayed increase in IL-7 levels during the progressive depletion of circulating CD4 T-cells as well as a dissociation between the acquisition of markers of T-cell effector differentiation and the loss of IL-7Rα expression, are distinctly observed in HIV-2 disease.

Patients and Methods

Studied populations

A cross sectional study involving 45 HIV-2 and 62 HIV-1 infected patients, currently living in Portugal and attending outpatient's clinics in Lisbon, with no evidence of ongoing opportunistic infections or tumours, was performed. The clinical and epidemiological data of the 2 cohorts, as well as of the healthy controls, are summarized in Table I. Additionally, a longitudinal study was performed in 9 HIV-2 infected patients. All subjects gave informed consent to blood sampling and processing and the study was approved by the Ethical Board of the Faculty of Medicine of Lisbon.

Table I: Clinical and epidemiological features [a]

	Healthy controls	HIV-2 infected patients [b]	HIV-1 infected patients [b]
Number (male/female)	50 (18/32)	45 (13/32)	62 (36/26)
Age (yrs)	41±2 (21-84)	48±2 (21-70)	37±1 (20-68)
Ethnicity			
Caucasian	44	23	40
Other	6	22	22
HIV transmission category			
Heterosexual	NA	35	37
Homosexual/bisexual	NA	2	13
Intravenous drug user	NA	1	8
Blood transfusion	NA	5	0
Other/Unknown	NA	2	4
CD3 count (cells/μL)	1692±84 (767-3488)	1408±91 (427-2629)*	1353±78 (261-3430)*
Percentage CD3 (%)	75±1 (58-86)	72±1 (48-87)	73±1 (44-94)
CD4 count (cells/μL)	1058±56 (476-2387)	585±53 (28-1436)**	441±39 (2-1919)** #
Percentage CD4 (%)	47±1 (33-65)	29±2 (2-48)**	24±1 (0.1-48)** #
CD8 count (cells/μL)	572±39 (166-1251)	765±64 (180-2203)*	832±57 (127-2210)*
Percentage CD8 (%)	25±0.9 (9-38)	40±2 (17-74)**	44±2 (13-86)**
Viremia (RNA, copies/ml)	NA	508±113 (200-4006) [c]	157251±56017 (50-3072400)#

[a] Data are mean ± SEM with limits in brackets, unless indicated otherwise. NA, not applicable.
[b] Statistical differences between a given HIV infected cohort and the healthy controls. *, $p<0.05$; **, $p<0.0001$. Statistical differences between HIV-1and HIV-2 infected patients. #, $p<0.05$.
[c] HIV-2 viremia was quantified and was <200 RNA copies/ml (cut-off) in 37 of the 42 patients studied.
[d] HIV-1 viremia was quantified and was <50 RNA copies/ml (cut-off) in one of the 62 patients studied.

Phenotypic and intracellular characterization of lymphocyte subsets

PBMC were isolated from fresh heparinized blood using Ficoll-Hypaque density separation gradient. PBMC were surface stained as previously described (34) with the following anti-human mAbs (clone specified in brackets): CD8 (RPA-T8), CD27 (M-T271), CD45RA (HI100), CD62L (SK11), CD31 (WM59), and mouse IgG1, IgG2b isotype controls (all FITC-conjugated), CD8 (RPA-T8), CD45RO (UCHL-1), CCR7 (3D12), CD62L (Dreg 56), and mouse IgG1 and IgG2a isotype controls (all PE-conjugated), CD3 (SK7), CD4 (SK3), CD8 (SK1) (all PerCP-conjugated), CD8 (RPA-T8), CD4 (SK3), CD45RA (HI100), and mouse IgG1 and -IgG2b isotype controls (all Allophycocyanin-conjugated), all from BD Biosciences (San Jose, CA); and IL7-Rα (40131.111) PE-conjugated from R&D Systems (Minneapolis, MN). For the intracellular Bcl-2 staining, cells were fixed with 2% formaldehyde and permeabilized with PBS/1% BSA/0.5% saponin before staining with FITC-conjugated mAb (Bcl-2/100, BD Biosciences). Fifty thousand events were acquired using a FACSCalibur flow cytometer and analysed using Cellquest software (BD Biosciences). Briefly, a lymphocyte gate was manually set using forward and side-scatter, and thresholds were set according to isotype-matched controls. Absolute numbers of lymphocyte subsets were found by multiplying their representation by the absolute lymphocyte count obtained at the clinical laboratory.

IL-7 Quantifications

IL-7 levels were quantified in serum using the high sensitivity IL-7 Quantikine HS ELISA kit (R&D Systems), according to manufacturer's instructions. Samples were assayed in duplicate.

Plasma Viral Load Assessment

HIV-1 viremia was quantified by RT-PCR (Ultrasensitive Test; detection threshold - 50 RNA copies/ml; Roche Molecular Systems, Branchburg, NJ). HIV-2 viral load was quantified using a RT-PCR based assay developed and performed by P.Gomes and M.H.Lourenço (42), which has a detection limit of 200 RNAcopies/ml. The cut-off value of the tests was considered for the purpose of the analysis in the cases where detection was below this level.

Quantification of Cellular proviral DNA load

Genomic DNA was extracted from 10^6 PBMC cells using AbiPrism 6100 Nucleic Acid Extractor (Applied Biosystems, Foster City, CA) according to the manufacture's instructions, and quantified using NanoDrop ND-100 spectrophotometer (NanoDrop Tecnologies, Wilmington, DE). Quantitative real-time PCR was performed in 50µl PCR mixture volume containing 25µl of Platinum Quantitative PCR SuperMix-UDG , 1µl ROX Reference Dye 50X , 5 mM of $MgCl_2$ (all from Invitrogen, Carlsbad, Ca), with 300 nM of each primer, 200 nM of TaqMan probe and 150 ng of DNA using a AbiPrism 7000 SDS (Applied Biosystems). Thermal cycling conditions were as follows: 2 min at 50 ^oC, 2 min at 95 ^oC, followed by 40 cycles for 15 sec at 95 ^oC and 1 min at 60 ^oC. HIV-1 and HIV-2 *gag* primers and FAM-MGB probes were designed in Primer Express 2.0 software (Applied Biosystems) and checked against the Los Alamos HIV database. The sequence of the primers and probes are as follows: Albumin Forward primer (AlbF): 5'- tgc atg aga aaa cgc cag taa - 3'; Albumin Reverse primer (AlbR): 5'- atg gtc gcc tgt tca cca a- 3'; Albumin Probe: 5'- FAM- tca cca aat gct gca cag a - MGB - 3'; HIV-1 Forward primer (HIV-1-2F): 5'- ggg aga att aga tcg atg gga aa - 3'; HIV-1 Reverse primer (HIV-1-2R): 5'- ctg ctt gcc cat act ata tgt ttt aat tta - 3'; HIV-1 Probe: 5'- FAM- ccc tgg cct taa ccg aat t - MGB - 3'; HIV-2 Forward primer (HIV-2-2F): 5'- cgc gag aaa ctc cgt ctt g - 3'; HIV-2 Reverse primer (HIV-2-2R): 5'- cac aca ata tgt ttt agc ctg tac ttt tt - ´3'; HIV-2 Probe: 5'- FAM - ccg ggc cgt aac ct - MGB - 3'. For each run, standard curves were generated from purified Albumin, HIV-1 *gag* and HIV-2 *gag* plasmids, ranging from 10^6 to 5 copies. Samples were run in duplicate and the input level of DNA was normalized to the albumin copy number. Data were expressed as number of HIV DNA copies per 10^6 PBMC.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA). The data are presented as arithmetic mean±SEM and were compared using analyses of variance (ANOVA) and unpaired *t* test. Pearson's correlation coefficient was used to assess the correlation between 2 variables. $p<0.05$ was considered to be significant.

Results

Strong correlation between the increase in circulating IL-7 and the CD4 depletion in HIV-2 infection

Serum IL-7 levels were significantly increased both in HIV-1 and HIV-2 infections as compared to healthy controls, and there were no significant differences between the two infections (Figure 1A). On the other hand, we found a strong correlation between the increased levels of circulating IL-7 and the degree of CD4 depletion ($p<0.0001$, $r= -0.5929$) in HIV-2+ patients, that contrasts with the absence of a significant correlation in our HIV-1 cohort (Figure 1B). This cohort, like the HIV-2 cohort, did not include a significant proportion of patients with extreme CD4 lymphopenia, which might explain the discrepancy between our data and the previous reports of a significant negative correlation between CD4 counts and IL-7 levels in HIV-1 infection. In these cases, the significance was usually reached due to the very high IL-7 levels exhibited by patients with less than 100 CD4 cells/μl (1, 14, 23-25).

In order to further clarify the relationship between IL-7 levels and disease progression, HIV-1 and HIV-2 infected patients were divided into three groups according to CD4 T-cell counts: >500 (early), 200-500 (intermediate) and <200 CD4 T-cells/μl (advanced). Although both HIV-2 and HIV-1 patients at intermediate and advanced stages of disease exhibited significantly higher IL-7 levels than healthy controls, increased IL-7 levels in early disease stage was only observed in HIV-1 infected patients (Figure 1C). Thus, the absence of elevated IL-7 in early disease may contribute to the strong correlation documented between IL-7 and CD4 lymphopenia in HIV-2 infection.

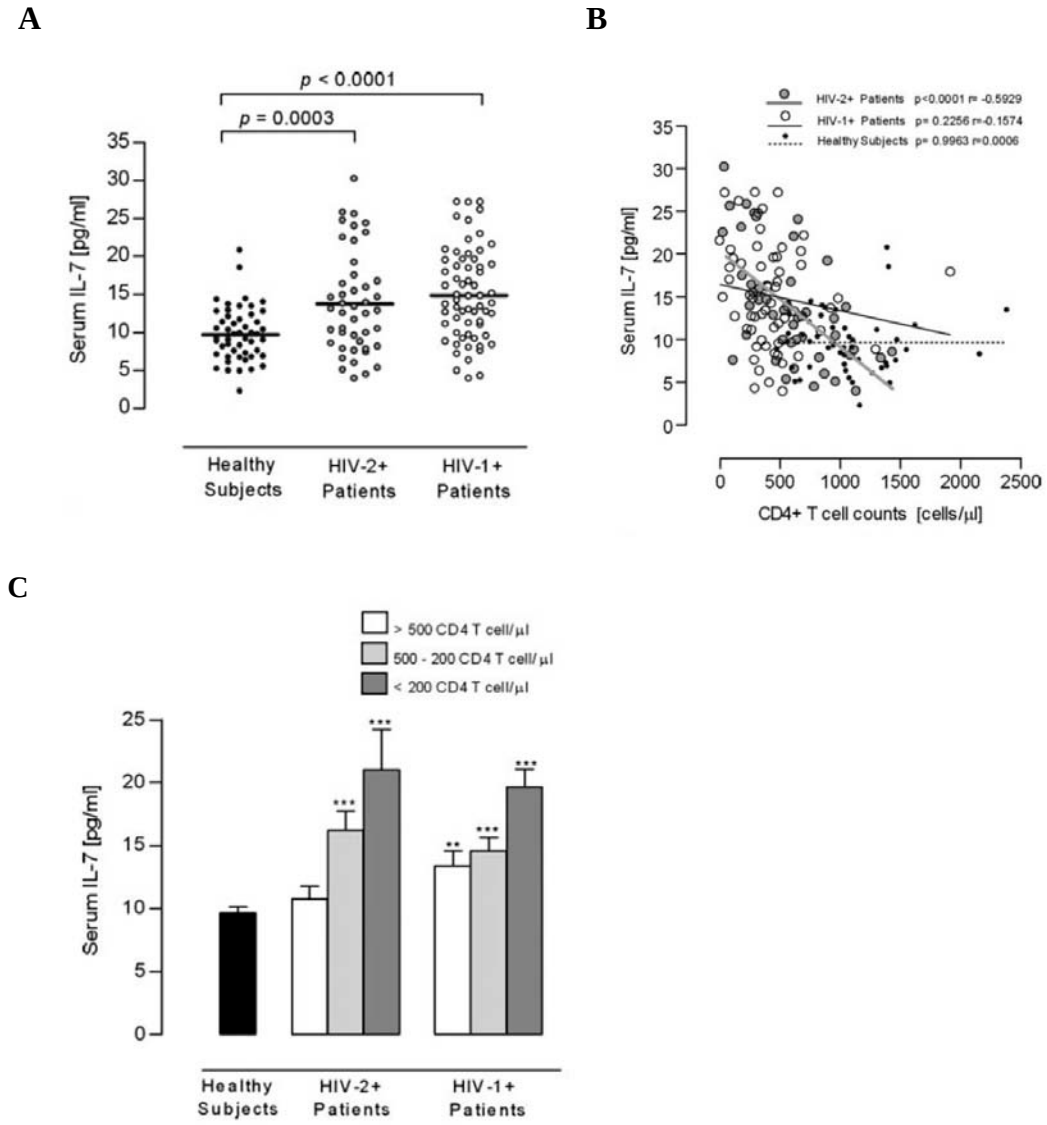

Figure 1 – Strong inverse correlation between circulating IL-7 levels and CD4 T-cell counts in HIV-2 infection.

(A) Serum IL-7 levels in HIV-1 and HIV-2 infected and healthy subjects were assessed by ELISA. Each dot represents one individual and bars represent means. **(B)** Correlation between serum IL-7 levels and CD4 T-cell counts in HIV-1 infected patients (open circle/black line), HIV-2 infected patients (grey circle/grey line) and healthy controls (black circle/dashed line). **(C)** Analysis of serum IL-7 levels in different sub-groups of HIV-1 and HIV-2 infected patients stratified according to CD4 T-cell counts. Bars represent mean±SEM. The overall differences were highly significantly p<0.0001 as assessed by ANOVA. Differences between two groups were compared by t tests. There were no statistically significant differences between HIV-1 and HIV-2 subgroups when comparing similar levels of CD4 depletion. Statistical significance between the different HIV infected groups as compared to healthy subjects: * $p<0.05$; ** $p<0.01$ and *** $p<0.001$.

The strong correlation between IL-7 and CD4 lymphopenia observed in HIV-2 infection is further supported by longitudinal studies of HIV-2 infected patients

A longitudinal analysis of circulating IL-7 levels and CD4 T-cell counts was performed in nine HIV-2 infected patients. As shown in Figure 2, low CD4 T-cell counts were associated with increased IL-7 levels in all patients. The period of follow-up ranged from 2 to 9 years. These untreated HIV-2 infected patients exhibited a reduced rate of CD4 decline as expected (32, 33, 36, 42). The graphs illustrate the consistency of the IL-7 measurements during follow-up and document changes in serum IL-7 levels inversely related to the alterations found in CD4 T-cell counts.

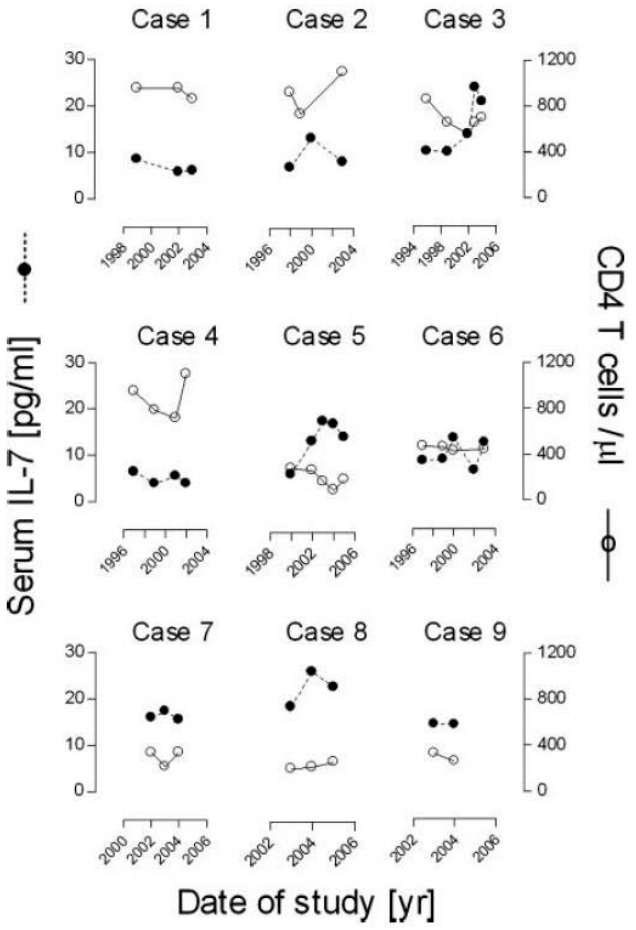

Figure 2 – Longitudinal analysis of circulating IL-7 levels and CD4 T-cell counts in HIV-2 infected patients.

Each graph illustrates the serum IL-7 levels (black circles) and the CD4 T-cell count (open circles) in each HIV-2 infected individual infected during disease follow-up.

No correlation of serum IL-7 levels with age

It has been suggested that serum IL-7 levels decreases with age (43, 44). Since HIV-2 infected patients tend to be older than HIV-1 infected subjects we looked for a possible impact of age on the circulating IL-7 levels and found no correlation between age and serum IL-7 in our three cohorts as illustrated in Figure 3A. Moreover, there were no statistically significant differences in IL-7 levels according to gender in the infected cohorts (p=0.1194 derived from the analysis of variance).

No significant correlation of serum IL-7 levels with viremia and proviral DNA

IL-7 is a powerful inducer of HIV-1 replication *in vitro* (45). However, there is conflicting data regarding the correlation between circulating IL-7 and HIV-1 viremia (1, 14). We found no correlation between serum IL-7 and HIV-1 viremia (r=0.0324, p=0.8094). Despite the lack of data on the ability of IL-7 to induce HIV-2 replication, it is unlikely that it will have a different effect to that reported for HIV-1 and SIV strains (45-47). Since the large majority of the HIV-2 infected patients had undetectable levels of viremia (below 200 RNA copies/ml), it was impossible to correlate this parameter with IL-7 levels.
In spite of the distinct viremia, HIV-1 and HIV-2 infected patients have been shown to have comparable levels of cell-associated viral load as assessed by proviral DNA (36, 48, 49). Accordingly, we found no significant difference between the levels of PBMC proviral DNA in the HIV-1 and HIV-2 cohorts. No correlation was found between circulating IL-7 and HIV-1 proviral DNA as well as with HIV-2 proviral DNA, as shown in Figure 3B.

Analysis of CD4 T-cell subsets / IL-7Rα expression in relation to circulating IL-7

We then investigated whether the apparent close association of increased IL-7 levels with CD4 depletion in HIV-2 infection had an impact on naive/memory imbalances and on IL-7Rα expression as assessed by flow cytometry on freshly isolated PBMC.
As illustrated in Figure 4A, a significant negative correlation was found between circulating IL-7 levels and naive CD4 T-cell counts in the HIV-2 cohort that was not observed in the HIV-1 cohort.

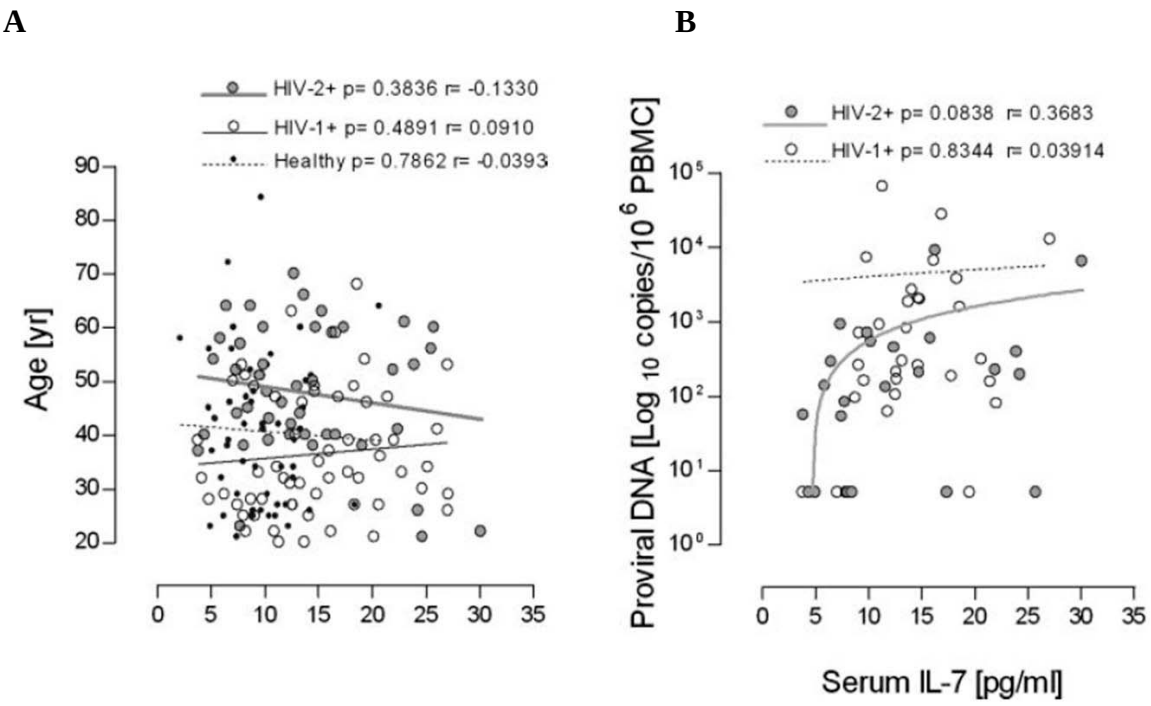

Figure 3 – No significant correlation between circulating IL-7 levels with age or virological parameters.

Correlation between serum IL-7 levels with: **(A)** age in healthy subjects as well as HIV-2 and HIV-1 infected patients and **(B)** PBMC associated proviral load quantified by real-time PCR in both HIV-2 and HIV-1 infected cohorts. Each dot represents one individual: healthy (black circle), HIV-1 infected (open circle) or HIV-2 infected (grey circle). The HIV-2 and HIV-1 cohorts evaluated do not differ significantly in terms of either CD4 counts or IL-7 levels.

The CD31 molecule has been proposed to be a marker for recent thymic emigrants as the $CD31^+$ subset of human naive T-cells is enriched in T-cell receptor excision circles (TREC) (50). When we looked for a correlation between IL-7 and the proportion of $CD31^+$ cells within the naive CD4 subset, the linear regression showed a similar marked positive slope both in HIV-2 and healthy cohorts that contrasts with the negative slope found in the HIV-1 cohort (Figure 4B). Moreover, this correlation reaches significance when the number of subjects is increased by the conjoint analysis of the HIV-2 and healthy cohorts (r= 0.3876; p= 0.0312). Although there were no significant differences between the frequency of $CD31^+$ cells within the naive CD4 subset in the three cohorts (HIV-2: 48.41±6.43; HIV-1: 43.87±3.57; and healthy: 42.50±3.11), the expected decline of this frequency with age was

not observed in HIV-2 infected patients (Figure 4C). Further studies are required to elucidate the relative contribution of the thymus and the periphery in the maintenance of this population, including TREC quantification. Despite this, our data suggest that the better preservation of this subset in HIV-2 than in HIV-1 infected patients is closely related to IL-7 levels.

Figures 4D and 4E illustrate the IL-7Rα expression levels within the naive and memory CD4 subsets, respectively. The HIV-2 patients included in this analysis were older than the HIV-1 patients (49±3 and 37±3 yr, respectively, p= 0.0058), but had similar CD4 counts and IL-7 levels. As expected, in all three cohorts there was a significant decrease in the levels of IL-7Rα ($p<0.006$), with cell differentiation defined by the loss of CD45RA. The HIV-1 infected patients exhibited a major reduction in the IL-7Rα expression as compared to healthy controls both in the naive and memory subsets, in agreement with the low levels of IL-7Rα expression in HIV-1 infected patients reported by others (24, 26, 27). However, in HIV-2 infected patients, there was only a significant decrease in IL-7Rα expression in the memory subset, suggesting maintenance of the IL-7Rα in the naive CD4 pool. The levels of CD4 T-cell activation as assessed by HLA-DR up-regulation were similar in the two infections (data not shown), suggesting that the discrepancies of IL-7Rα expression cannot be attributed to different states of T-cell activation.

The ability of IL-7 to promote T-cell survival has been shown to be related to the up-regulation of the antiapoptotic molecule Bcl-2 (3). We measured intracellular Bcl-2 by flow cytometry on freshly isolated PBMC, and, despite an apparent trend in HIV-2 infected patients for higher MFI (median intensity of fluorescence) within the CD4 subset, no significant differences were found in the three cohorts (Figure 4F).

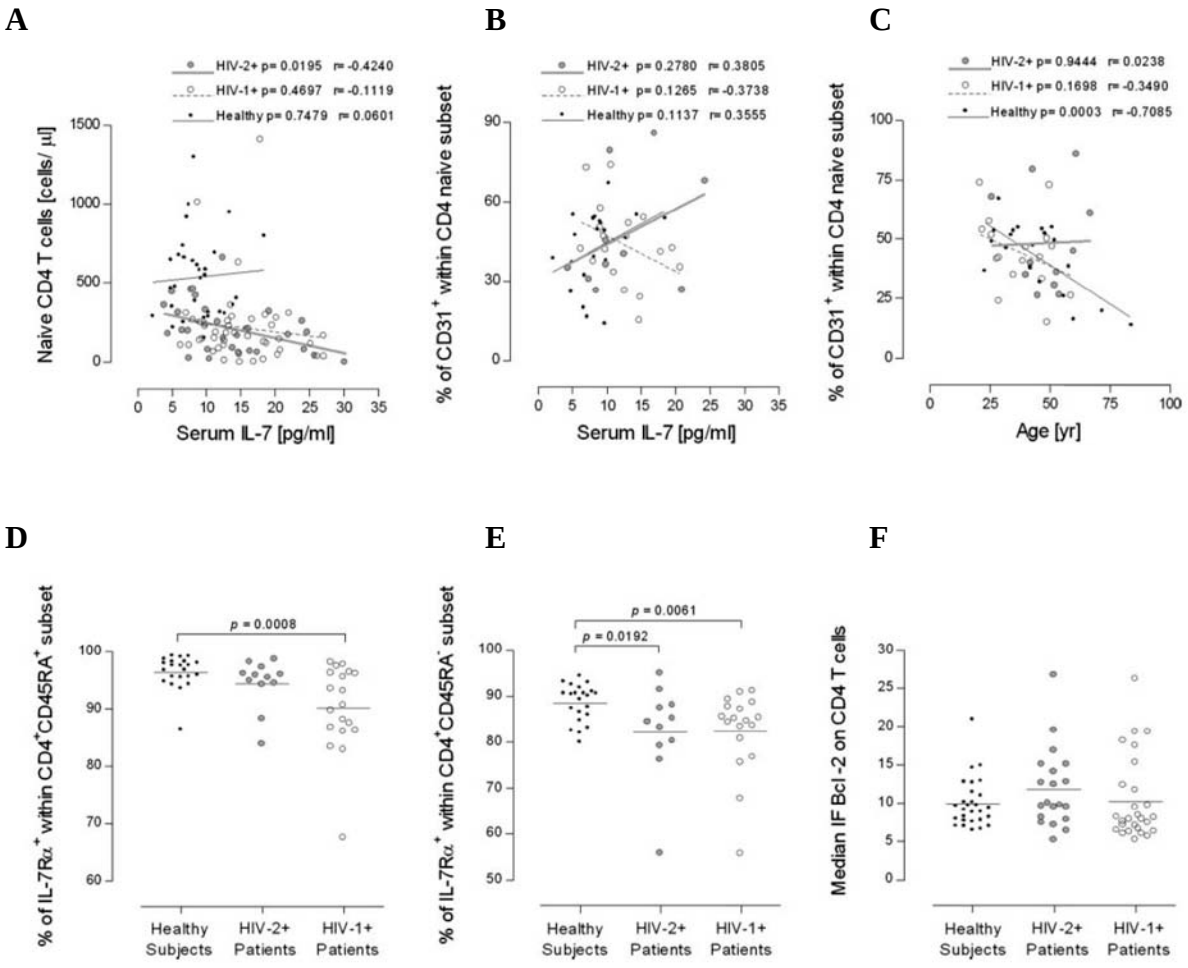

Figure 4 – Analysis of CD4 T-cell subsets and IL-7Rα expression

PBMC from HIV-1 and HIV-2 infected patients, as well as healthy controls were phenotypically characterized using 4 color flow cytometry. **(A).** Correlation between serum IL-7 levels and circulating naive CD4 T-cell counts, as defined by the simultaneous expression of CD45RA and CD62L. The naive subset was further characterized in terms of the expression of CD31 and the correlation between the proportion of $CD31^+$ cells within the CD4 naive subset and serum IL-7 levels **(B)** and age **(C)** was analyzed. Frequency of IL-7Rα^+ cells within the naive **(D)** and the memory **(E)** CD4 subsets; the overall differences were significant as assessed by ANOVA (p=0.001 and p=0.022, respectively), and the significant p values of the differences between two groups evaluated by t test are shown. **(F)** Median fluorescence intensity of intracellular Bcl-2 within CD4 T-cells. Each dot represents one individual: HIV-1 infected patients (open circle), HIV-2 infected patients (grey circle) and healthy controls (black circle). Bars represent means. The studied HIV-2 and HIV-1 cohorts had comparable CD4 counts and IL-7 levels.

Analysis of CD8 T-cell subsets / IL-7Rα expression in relation to circulating IL-7

$CD8^+$ T cell differentiation was assessed by the expression of CD27, CCR7 and CD45RA in cohorts of HIV-2 and HIV-1 infected patients with comparable degrees of CD4 counts and IL-7 levels but differing viremia. We found similar levels of naive and central memory CD8 T-cell pool depletion in both infections (Figure 5A and 5B, respectively). However, HIV-1 infected patients exhibited an expansion of $CD8^+$ T-cells with an intermediate-differentiated phenotype $CD45RA^-CCR7^-CD27^+$, which was not found in the HIV-2 cohort (Figure 5C). In fact, the major CD8 expansion observed in HIV-2 infected patients was essentially due to terminally-differentiated effector cells $CCR7^-CD27^-CD45RA^+$ (Figure 5D). These results are in agreement with our previous data from other HIV-2 and HIV-1 cohorts where CD8 differentiation was assessed by CD62L, CD28 and CD27 expression as well as IL-2 and/or IFNγ production (34, 38). No significant correlations were found between serum IL-7 levels and the frequency of the different CD8 subsets in either infection.

All the three cohorts exhibited a progressive loss of expression of IL-7Rα in the CD8 subpopulations as they acquired a more differentiated phenotype. However, HIV-2 and HIV-1 infected patients exhibited significantly lower expression levels of IL-7Rα in all the CD8 subsets as compared to healthy controls (Figure 5E). When we compared both infected cohorts, the HIV-2 infected patients preserved significantly higher levels of IL-7Rα expression than the HIV-1+ patients in all the CD8 effector-memory subpopulations (Figure 5E). A similar level of CD8 activation assessed by CD38 and/or HLA-DR was observed in the two infected cohorts (data not shown), and, therefore, it is unlikely that the discrepant levels of IL-7Rα expression in the two infections were due to differences in the cell-activation state.

In summary, the expanded CD8 T-cell pool, observed in both HIV cohorts, showed skewing towards a terminally differentiated effector phenotype paralleled by a better preserved expression of IL-7Rα in the HIV-2+ as compared to the HIV-1+ individuals.

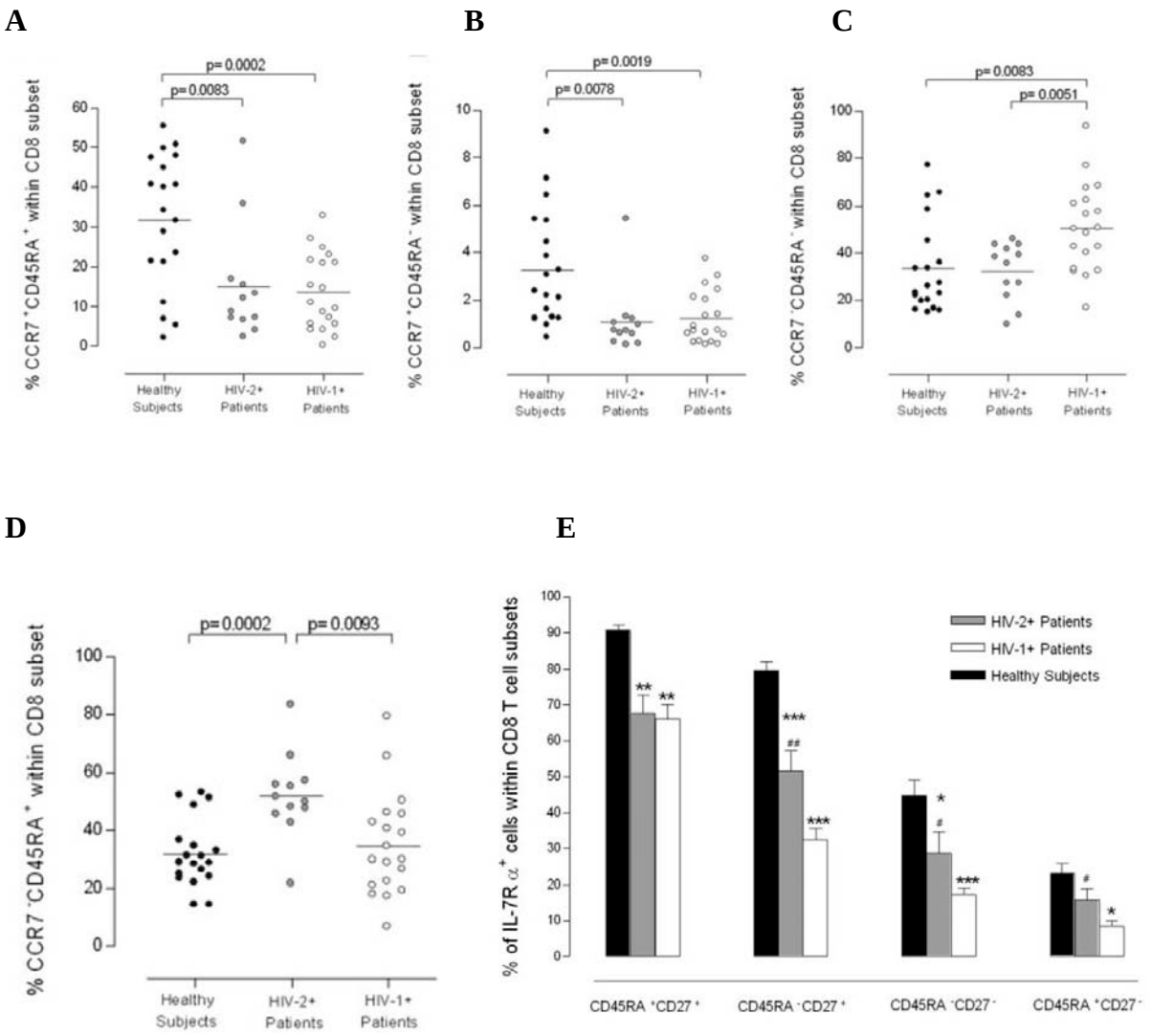

Figure 5 – Imbalances of CD8 T-cell subsets and IL-7Rα expression.

CD8 T-cell subsets were defined by the simultaneous analysis of CD45RA and CCR7 or CD27 by flow cytometry using freshly isolated PBMC. Analysis of the frequency of naive $CCR7^{+}CD45RA^{+}$ **(A)**, central-memory $CCR7^{+}CD45RA^{-}$ **(B)**, intermediate $CCR7^{-}CD45RA^{-}$ **(C)**, and terminally-differentiated $CCR7^{-}CD45RA^{+}$ **(D)** within the CD8 T-cells. Each dot represents one individual: HIV-1 infected (open circle), HIV-2 infected (grey circle) and healthy (black circle). Bars represent means. **(E)** IL-7Rα expression was assessed within successive subsets of CD8 T-cell differentiation, namely, $CD45RA^{+}CD27^{+}$, $CD45RA^{-}CD27^{+}$, $CD45RA^{-}CD27^{-}$ and $CD45RA^{+}CD27^{-}$ in 18 HIV-1 infected patients, 12 HIV-2 infected patients, and 22 healthy controls. The studied HIV-2 and HIV-1 cohorts had comparable CD4 counts and IL-7 levels. The overall differences between the groups were statistically significant $p<0.005$ to $p<0.0001$ assessed by ANOVA. Bars represent mean±SEM. Statistical significance between the different HIV infected groups as compared to healthy subjects: * $p<0.05$; ** $p<0.01$ and *** $p<0.001$ and between HIV-1 and HIV-2 infected patients: # $p<0.05$ and ## $p<0.01$ as assessed by t test.

Discussion

This is the first study evaluating both the levels of circulating IL-7 and the expression of the IL-7Rα in patients infected with the second AIDS-associated virus, the HIV-2. HIV-2 disease has several unique features that make it especially attractive to address the role of IL-7 in lymphopenic clinical settings. Firstly, it is characterized by a progressive decrease in CD4 counts associated with pan-immuneactivation, though at a much slower rate than that observed in HIV-1 infection (30, 32-34). Secondly, in contrast with HIV-1, HIV-2 infection is associated with reduced viremia at all disease stages (33, 34, 36-38). Thirdly, it has a relatively favorable clinical outcome with limited impact on the mortality of the majority of the infected adults (30-32), suggesting that HIV-2 infected patients somehow retain the capacity to replace and sustain numbers of immunologically competent CD4 T-cells.

Here, we describe an increase in circulating IL-7 levels in strong correlation with the degree of $CD4^+$ T-cell depletion. This was documented in a cross-sectional study involving HIV-2 patients with different levels of CD4 counts without known ongoing opportunistic infections, as well as in a longitudinal study. This cohort was compared with an HIV-1 infected cohort, similarly underrepresented by profoundly lymphopenic patients, which may explain the absence of this correlation in contrast to the majority of the HIV-1 studies reported in the literature (1, 14, 23-25).

The mechanisms driving increased circulating IL-7 levels remain unclear (1, 16). Two non-mutually exclusive explanations have been proposed.

Elevated IL-7 levels may result from increased production in response to the CD4 lymphopenia (1, 14, 15). This would require a more marked CD4 loss in HIV-1 than in HIV-2 infected patients despite the same levels of peripheral blood CD4 counts, in order to explain the disease specific differences in the kinetics of IL-7 increase in early infection. We found similar levels of circulating IL-7 in early HIV-2 disease and healthy subjects, in contrast to the significant increase found in early HIV-1 infection. However, there is cumulative evidence that during early HIV-1 disease the peripheral blood compartment overestimates the degree of CD4 depletion of the body due to the traffic alterations that promote lymphocyte retention in the lymphoid tissue as illustrated through the study of tonsils and lymph nodes

(51, 52). Yet, recent data show a marked depletion of $CD4^+$ T-cell subsets in the gut, occurring during acute HIV-1 infection and persisting throughout disease (53-55). There are no data on lymph node or mucosal pathology during HIV-2 infection, but it is reasonable to speculate that the establishment of HIV-2 infection may not be associated with a high viremia peak and major depletion of the memory compartment, given the absence of clinical reports of HIV-2 acute infection. The possible contribution of gut-associated CD4 depletion in triggering the early increase of IL-7 production in HIV-1 infection deserves further exploration.

Alternatively, the circulating IL-7 levels may increase as a result of its diminished adsorption by a reduced number of cells expressing the IL-7Rα (16). Our findings support this possibility given the reduced levels of IL-7Rα expression in HIV-1 as compared to HIV-2 infected patients with the same degree of CD4 depletion. Although, IL-7 consumption has been shown to transiently induce IL-7 down-regulation, the high IL-7 levels observed in HIV-1 infection argue against this explanation. The different IL-7Rα expression in the two infections is particularly relevant in view of the similar state of immune-activation as assessed by the expression of HLA-DR and CD38 within both CD4 and CD8 T-cell subsets, in agreement with our previous studies in other cohorts using a larger panel of activation markers (34, 56). Moreover, several factors would favor a lower level of expression of the IL-7Rα in HIV-2 infection, namely: HIV-2 infected patients were shown to be older, and, ageing is associated with impaired IL-7Rα expression (22); the longer length of the infection in the case of HIV-2; and the terminally differentiated profile exhibited by the CD8 T cells of these patients.

IL-7 administration to SIV infected non-human primates has been shown to expand naive and memory T cell subsets (6, 9, 10). Although the frequency of naive and central memory T-cells were not higher in HIV-2 as compared to HIV-1 infected patients with the same degree of CD4 depletion, it is possible that a well adjusted and balanced increase in circulating IL-7 levels may contribute to the slower rate of loss of these populations in HIV-2 infection. Moreover, almost all subsets of CD4 and CD8 T cell, irrespectively of their differentiation state, were shown to have higher levels of IL-7Rα expression in HIV-2 than in HIV-1 infected patients, possibly indicative of a higher proliferative capacity or survival in response to IL-7. The preserved expression of the IL-7Rα, in particular within the CD4 naive T-cells,

could also lead to an increased consumption of IL-7 in HIV-2 as compared to HIV-1 infection, and partly explain the delayed increase in circulating IL-7 levels.

On the other hand, IL-7 is a strong inducer of HIV-1 replication (45, 57, 58), leading to its proposed use as a therapy to purge the viral reservoirs in HIV-1 infected patients virally suppressed by ART (45). Although there are no data on the ability of IL-7 to promote HIV-2 replication, it would be expected to be comparable given the similarity of the two viral promoter regions (LTRs) (59). No differences were found between the levels of HIV-2 and HIV-1 proviral DNA in PBMCs, in agreement with other studies (36, 48, 56). The low to undetectable HIV-2 viremia in this context further emphasizes the efficiency of the ill-defined mechanisms involved in the control of viral replication in HIV-2 infected patients in the absence of antiretroviral therapy.

In summary, this comparison of two persistent infections associated with progressive CD4 depletion and immune-activation demonstrates the better prognosis is not necessarily associated with higher levels of IL-7. Moreover, the delayed increase in IL-7 levels, coupled with sustained expression of IL-7Rα suggests a maximization of available resources. The observation that increased IL-7 levels early in HIV-1 infection seem unable to reduce the rate of CD4 loss, and the impaired expression of the IL-7Rα irrespective of the state of cell differentiation raises concerns regarding the use of IL-7 therapy in HIV-1 infection.

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EFFICIENT THYMOPOIESIS CONTRIBUTES TO THE MAINTENANCE OF PERIPHERAL CD4 T CELLS DURING CHRONIC HUMAN IMMUNODEFICIENCY VIRUS TYPE 2 INFECTION.

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Abstract

Human immunodeficiency virus type 2 (HIV-2) infection leads to a lifelong asymptomatic period in the majority of patients. Even in patients with progressive disease, a slow CD4 count decline characterizes the chronic phase of HIV-2 infection, suggesting that peripheral T-cell homeostasis is controlled better following HIV-2 infection than following HIV-1 infection. Herein we showed that, in contrast to HIV-1-infected patients, HIV-2-infected patients demonstrate enhanced thymic function compared to age-matched healthy individuals. The correlation between higher thymic production and lower CD4 T-cell loss in these patients suggests that efficient thymopoiesis is implicated in the long-lasting maintenance of CD4 T-cell counts in HIV-2 disease.

Human immunodeficiency virus type 2 (HIV-2) infection is associated with a more benign course of disease than HIV-1 infection is. The majority of HIV-2-infected individuals remain asymptomatic for years following infection and their disease behaves like that of HIV-1-infected long-term nonprogressors, while the patients who progress to disease exhibit a much slower rate of CD4 decline than the rate for HIV-1-infected patients (9, 12, 21). The latter group of HIV-2-infected patients is reminiscent of HIV-1 slow progressors, characterized by a slow CD4 T-cell decline despite detectable viremia (3, 13). Furthermore, studies in geographic areas where high HIV-2 prevalence is observed demonstrated that the life expectancy for individuals with HIV-2 infection is often close to that of uninfected individuals living in the same villages (12).

The factors determining the delayed disease progression in HIV-2 infection remain largely unknown (14). Although plasma viral load is much lower in HIV-2 infection than in HIV-1 infection (2), the proviral loads of HIV-2-infected patients are similar to those of HIV-1-infected patients at the same disease stage (10, 11), suggesting that the two viruses do not differ significantly in their ability to establish infection and replicate in human cells (1, 17). On the other hand, similar degrees of T-cell hyperactivation, as well as increased T-cell cycling, were observed for both HIV-1- and HIV-2-infected patients with similar degrees of CD4 depletion (18). This suggests an intimate link between generalized immune activation and associated cell death and CD4 depletion in both infections despite distinct viremias and clinical outcomes.

HIV-1 infection is associated with an impairment of intrathymic precursor T-cell proliferation resulting in a huge reduction of de novo T-cell production thought to participate in the progressive decline of peripheral CD4 T-cell counts, in particular in the recent thymic emigrants (RTEs) and naive T-cell compartments (4). An imbalance between production and destruction of peripheral CD4 T-cells would lead to their progressive decline over time and eventually to AIDS (5, 6). Moreover, in slow progressor HIV-1-infected patients, we recently demonstrated that the maintenance of circulating CD4 T-cells is strongly associated with efficient thymopoiesis (3).

We thus hypothesized that the maintenance of de novo T-cell production may counteract the peripheral CD4 loss that is known to occur in HIV-2 infection and represents a major mechanism underlying the slow progression of HIV-2 disease. Here we analyzed the role of thymopoiesis in the long-term maintenance of peripheral $CD4^+$ T-cell numbers in a cohort of untreated chronically HIV-2- and HIV-1-infected subjects currently living in Portugal and attending outpatient clinics in Lisbon, Portugal, compared to age-matched healthy individuals. A summary of the clinical features of the patients studied and healthy controls is shown in table 1.

Table 1. Characteristics of the HIV-1- and HIV-2-infected patients and healthy controls studied

Characteristic	Value for group [a]					
	Controls		HIV-1-infected patients		HIV-2-infected patients	
	35 to 45 yr	>45 yr	35 to 45 yr	>45 yr	35 to 45 yr	>45 yr
No. of subjects	11	12	6	9	7	13
Ethnicity [b]	9 C; 2 A	11 C; 1 A	5 C; 1 A	6 C; 3 A	4 C; 3 A	7 C; 6 A
Age (yr) [c]	39 (36-45)	54 (46-65)	41 (35-43)	54 (46-65)	38 35-45)	49 (46-68)
Time (mo) after first Seropositivity [c,d]	NA	NA	ND	ND	19 (7-133)	18 (2-172)
Viral load (log copies/ml)[c]	NA	NA	4.06 (2.63-4.98)	4.55 (3.28-5.41)	<200 [e]	<200 [e] (<200-4006)
Absolute CD4 count (cells/ml) [c]	920 (535-1867)	882 (347-1758)	291** (92-704)	240** (88-986)	927 (333-1184)	593* (220-830)
Absolute CD8 count (cells/ml) [c]	336 (105-1236)	265 (99-770)	654* (269-2007)	781** (518-2210)	845* (611-1778)	670** (202-2180)

[a] The subjects are grouped by disease status (controls and HIV-1- and HIV-2-infected patients) and age. NA, not applicable; ND, not determined. Statistical differences between the values for HIV-infected patients and age-matched controls (Mann-Whitney test) are indicated as follows: *, $P < 0.05$; **, $P < 0.01$.
[b] The number of Caucasians (C) and Africans (A) are shown (all the African subjects had been residing in Europe for several years at the sampling time).
[c] The median value is shown, and the range is shown in parentheses.
[d] All the HIV-2-infected individuals were identified as seropositive at their first visit. (The HIV-1 patients were sampled for another cross-sectional study.)
[e] HIV-2 viremia was quantified by a reverse transcriptase PCR-based test; all but one of the HIV-2 infected patients had a viral load below 200 RNA copies/ml (cutoff value).

Thymic activity was estimated by measurement of the intrathymic proliferation history of circulating RTEs by quantification of the signal joint/beta T-cell receptor excision circle (sj/ βTREC) ratio as described previously (3, 4, 20). This marker, which is independent of peripheral T-cell proliferation and death rate, directly reflects the number of proliferation cycles undergone by precursor T cells during their intrathymic differentiation and thus thymic output (4).

Surprisingly, while in both HIV-1 and HIV-2 patients, the younger individuals (35 to 45 years old) demonstrate a low thymic function (median sj/ βTREC ratios of 11.3 and 6.5 for HIV-1 and HIV-2 patients, respectively, compared to a ratio of 24.1 in the healthy control group [$P = 0.026$ and $P = 0.02$, respectively]; Fig. 1A), a significantly higher sj/βTREC ratio characterizes HIV-2-infected subjects that were > 45 years old compared to healthy controls (median sj/βTREC ratio of 17.0 in HIV-2-infected patients compared to 4.9 in control individuals [$P = 0.0047$]; Fig. 1A). This contrasts with the expected low intrathymic proliferation observed in age-matched HIV-1-infected patients (sj/βTREC ratio of 6.9). In fact, analysis of the sj/βTREC ratio as a function of age shows that the expected age dependence of thymic output is not observed in the HIV-2-infected patients (Fig. 1B). While most patients with chronic HIV-1 infection demonstrate a rapid and persistent defect in thymic output (3, 4), the impairment of thymopoiesis observed in the younger groups of HIV-2-infected patients is not further exacerbated by aging. In contrast, it seems that this function is maintained in this group of patients, so that in aged individuals, the sj/ βTREC ratio becomes greater than in healthy individuals, most likely leading to long-term sustained thymic output.

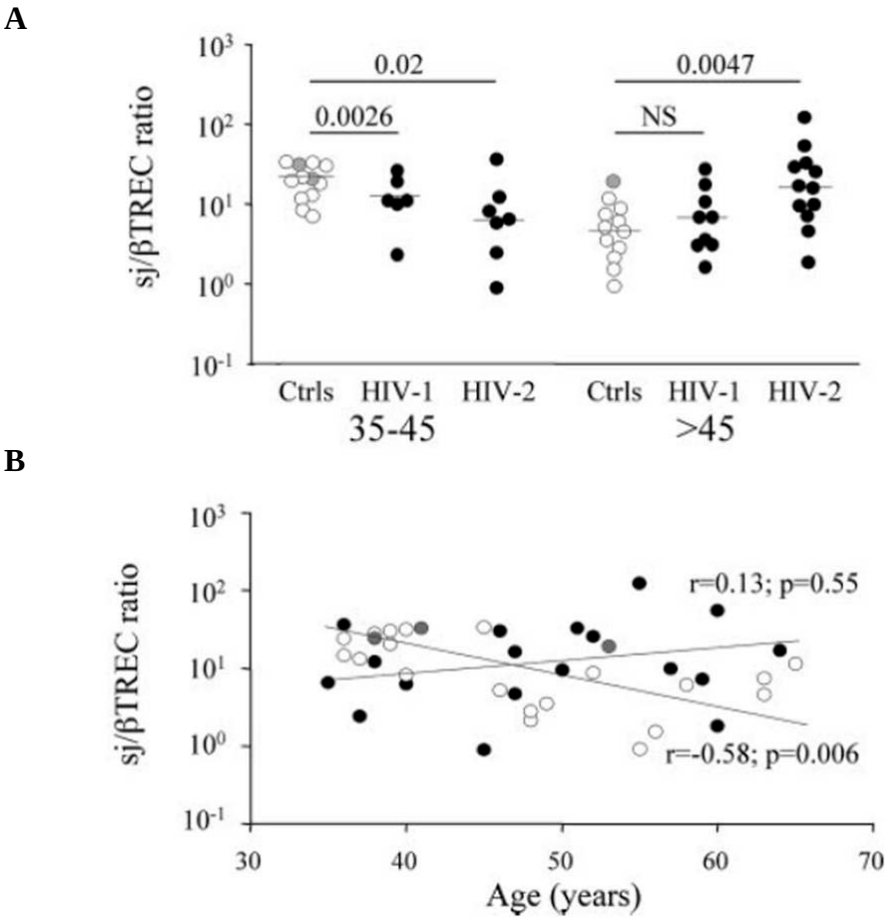


Figure 1 – HIV-2-infected patients demonstrate high thymic function.

(A) The sj/βTREC ratio, a measure of thymic activity, was calculated for healthy individuals (controls [Ctrls]) and HIV-1- and HIV-2-infected patients. The three groups were subdivided into groups by age (35 to 45 years old and > 45 years old). Gray symbols represent healthy control individuals of African origin. Statistical differences (*P* values) between the different groups are shown above the values (Mann- Whitney test). NS, not significant. **(B)** Correlation between the sj/βTREC ratio and age in healthy controls (white and gray symbols, representing Caucasian and African individuals, respectively, and HIV-2-infected patients (black symbols). Spearman's correlation and associated probability are shown for the control group.

Nevertheless, one must consider that the assessment of sjTREC frequency may be influenced by variable degrees of immune activation/proliferation in the groups studied. We thus also determined the sjTREC concentration (sjTREC/ml of blood) which, although influenced by thymic output and RTE survival, does not depend upon the general state of immune activation/proliferation (7, 8).

Interestingly, the analysis of the sjTREC concentration also showed that the patients that were >45 years old behave like age-matched controls do, with a significant reduction characterizing 35- to 45-year-old individuals (median sjTREC/ml of 670 and 1,821 in HIV-2-infected patients and controls, respectively; $P = 0.02$) (Fig. 2B). The parallelism between sjTREC frequency and concentration (Fig. 2A and B) suggests that the proliferation of TREC-positive cells is not of major importance in the regulation of naive T-cell counts during HIV-2 infection. However, HIV-2- and HIV-1-infected patients with equivalent degrees of CD4 depletion demonstrate similar enhancement of immune activation levels (18, 19). It is thus most probable, as demonstrated by Sieg et al. in HIV-1 infection that T cells expressing activation/proliferation markers (CD69, HLA-DR, Ki-67. . .) in HIV-2 infection are also determined to die rapidly *in vivo* (15, 16). This is further emphasized by analyzing the relationships between the sj/βTREC ratio (thymic production) and the sjTREC concentration (approximate RTE counts). As expected, both parameters correlated nicely in the control group and the HIV-1-infected patients ($r = 0.574$ and $P = 0.006$ and $r = 0.653$ and $P = 0.014$, respectively [Fig. 2C, left graph]). In contrast, in HIV-2-infected patients, thymic production was not associated with RTE concentration (Fig. 2C, right graph), demonstrating that the sustained thymic function in older HIV-2-infected patients does not lead to the expected enlargement of the TREC-rich T-cell population, likely being concomitantly expanded by increased cell death and/or accelerated maturation. This is further emphasized by the absence of correlation between circulating interleukin 7 levels and sjTREC quantification in HIV-2-infected patients (1).

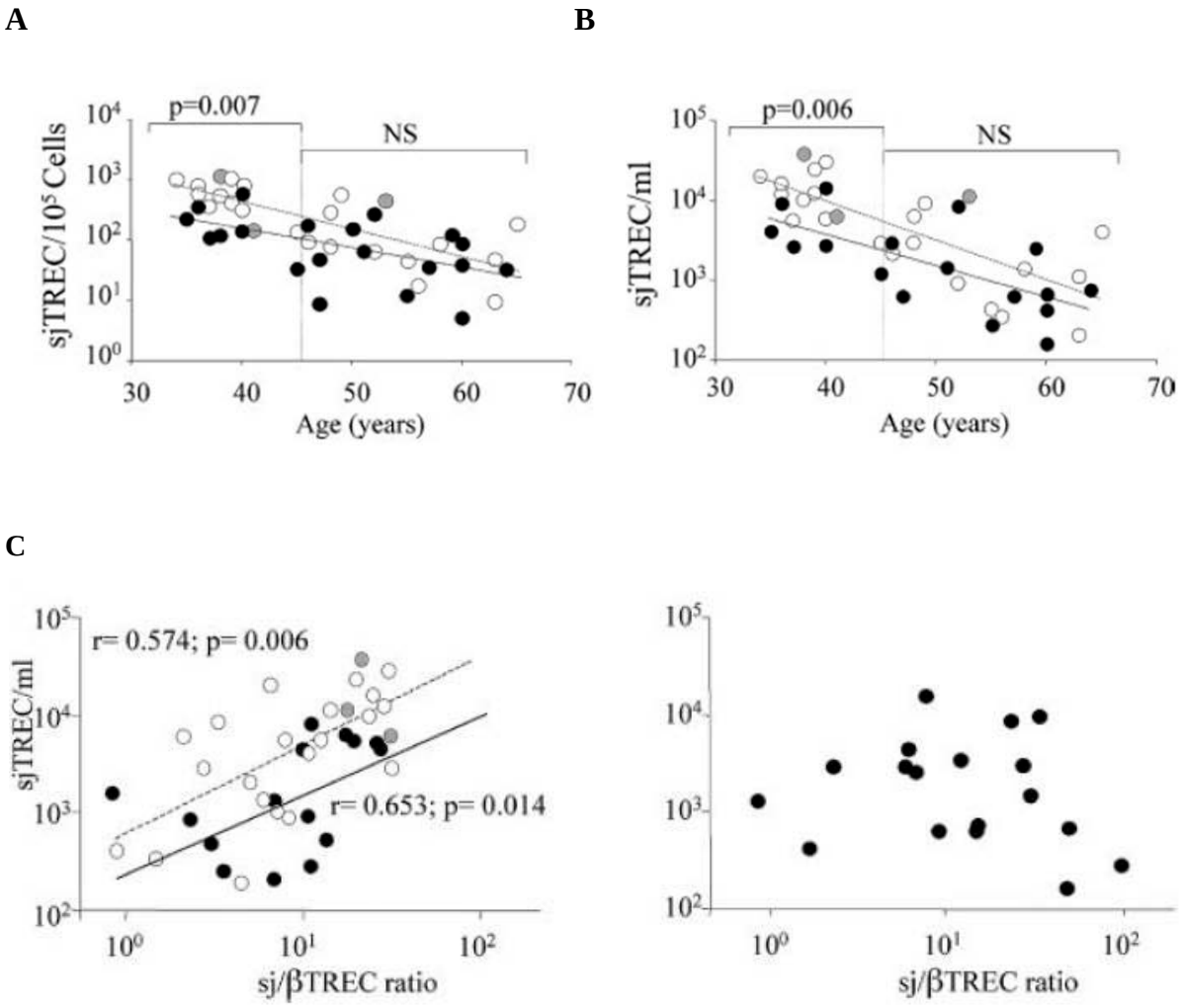
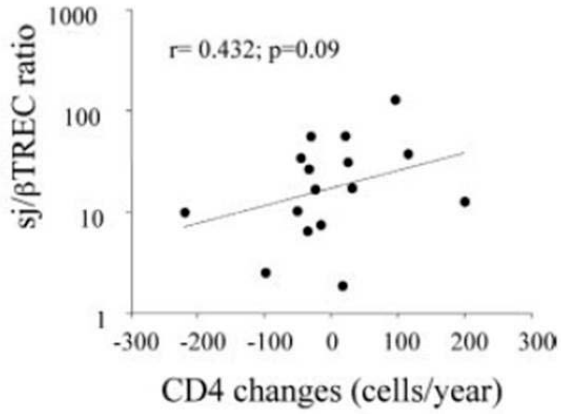


Figure 2 – Consequences of the increased thymic function on peripheral RTE population.

(A) Relationships between the sjTREC frequency and age in HIV-2-infected patients and healthy control individuals. **(B)** Relationships between sjTREC concentration and age in HIV-2-infected patients and healthy control individuals. In both panels A and B, the white symbols and dashed lines represent healthy controls (gray dots represent healthy controls of African origin), and the black symbols and solid lines represent the HIV-2-infected patients. The *P* values from the statistical analysis (Mann-Whitney test) for each age group are shown above the values. NS, not significant. **(C)** Relationships between the sjTREC concentration and thymic function in healthy controls (white and gray symbols, representing Caucasian and African individuals, respectively), HIV-1-infected patients (left graph, black symbols), and HIV-2-infected patients (right graph). When significant, *P* values from the statistical analysis are shown (Spearman's correlation and associated probability).

In order to estimate the impact of the observed enhanced thymopoiesis on the relative resistance to disease in the HIV-2-infected patients, we calculated the rate of CD4 count decline in this group of patients over a median period of 2.5 years preceding the analysis of thymic function. In HIV-2-infected patients, the sj/βTREC ratio tends to correlate with the variations in CD4 T-cell counts over time (r = 0.432 and P = 0.09; Fig. 3A). Such a correlation does not exist in the HIV-1-infected patients (r = 0.06 and not statistically significant). Interestingly, when classifying the patients according to their sj/βTREC ratio, a significant difference was observed in their capacity to maintain CD4 T-cell counts over the follow-up period. While patients demonstrating efficient thymopoiesis (i.e., sj/βTREC ratio of >10) preserve their CD4 T-cell counts over the follow-up period (median CD4 change of +25 cells/ year [range, -43 to +202]; Fig. 3B), patients with low sj/βTREC ratio (<10) show a slow but definite decline in circulating CD4 T-cell numbers (median loss of -40 cells/year [range, -217 to +19; P = 0.023]). In contrast, in the HIV-1- infected group, the extent of intrathymic precursor T-cell proliferation does not correlate with CD4 T-cell decline (median CD4 changes of -122 and -73 cells/year in patients with sj/βTREC ratios of <10 and >10, respectively; not statistically significant; data not shown).

A



B

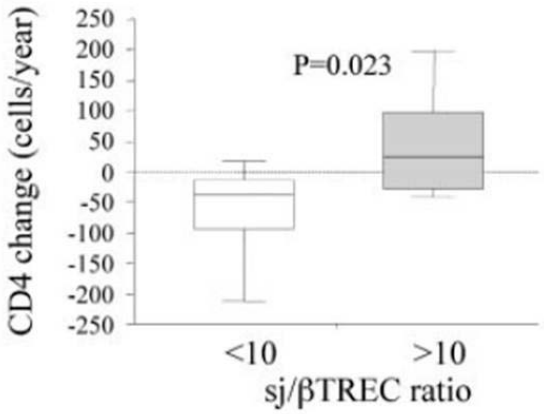


Figure 3 – Increased thymic output translates into maintenance of CD4 counts.

(A) Relationships between the sj/βTREC ratio and CD4 changes over time in HIV-2-infected patients. The correlation coefficient (Spearman's test) and associated probability are shown. **(B)** CD4 changes in HIV-2-infected patients with sj/βTREC ratios of <10 and ≥10. CD4 change values were calculated using the slope of CD4 counts measured over a period of >2.5 years before sampling. Statistical differences between the two groups (*P* values) are shown above the ratios (Mann-Whitney test).

Taken together, these data demonstrate that patients with chronic HIV-2 infections maintain thymic production for prolonged periods of time, even after they reach 45 years of age, when significant thymic involution is observed in healthy individuals. This effect, reminiscent of what was observed in HIV-1-infected slow progressors, suggests that increased CD4 T-cell death can, at least partly, be compensated for by an overproduction of new T cells in HIV-2-induced pathology. The fact that younger HIV-2-infected patients demonstrate a reduced thymic function suggests either that HIV-2 infection can lead to various levels of pathogenesis or that sustained thymic function in aging patients is a mechanism that develops slowly to compensate for increased cell death. Patients who cannot maintain thymic function would exhibit progressively lower CD4 T-cell counts, eventually leading to AIDS, while others whose thymus remains functional maintain CD4 counts and remain asymptomatic for several decades. However, it is possible that efficient thymopoiesis is both a cause and consequence of limited pathogenicity in HIV-2. It is quite possible that low viral loads in HIV-2-infected patients, by inducing limited homeostatic perturbations, leads to maintained thymic potential and that when lymphopenia occurs, this capacity of the thymus to produce new T cells allows the maintenance of CD4 counts and naive T-cell diversity sufficiently high to limit progression of the disease.

Interestingly, Poulsen et al. observed that in some West African villages, older people (55 to 80 years of age) with HIV-2 infection have the same mortality risk as uninfected individuals do (12). The capacity of these patients to maintain de novo T-cell production through efficient thymopoiesis despite aging may participate to their longevity in the presence of HIV-2 infection.

These new data on the role of the thymus in this natural model of attenuated HIV infection bring new arguments to the contribution of ongoing thymopoiesis for HIV pathogenesis and the rate of progression to AIDS and strengthen the importance of the thymus as a target for immune- based therapies.

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Conclusions and Future Perspectives

HIV-1 infection is characterized by a progressive depletion of CD4 T cells that cannot be solely attributed to direct viral killing (1). The persistent and generalized immune activation contributes to a greater susceptibility of lymphocytes to apoptosis and/or anergy, and is thought to increase the outflow of naive T cells into the memory-effector compartment (1, 2). On the other hand, *de novo* T cell production in the thymus is impaired by HIV-1 infection (2). The maintenance of the naive T cell pool is known to be due to homeostatic mechanisms of proliferation of peripheral T cells combined with an age-dependent contribution of thymic output. HIV-1 can directly and indirectly influence the production of various cytokines that regulate T cell homeostasis (3). Also, HIV-1 infects thymocytes and hematopoietic precursor cells and promotes the disruption of the thymic microenvironments necessary for the differentiation of T cells (4).

HIV-2 infection is associated with a much slower progressive CD4 T cell decline than HIV-1 infection. However both infections show similar levels of hyperimmune activation in parallel with CD4 depletion, throughout the natural history of the disease, despite the reduced viremia that characterizes HIV-2 infection (5). Thus, it is reasonable to expect that HIV-2 infected individuals have a better preserved ability of T cell replenishment. HIV-2 was used in this work, as a model of attenuated HIV disease, to provide insights into the alterations of the mechanisms necessary for the maintenance of the T cell pool during HIV pathogenesis.

The role of the IL-7 network in lymphopenic clinical settings was addressed through the evaluation of both the levels of circulating IL-7 and the expression of the IL-7Rα in CD4 and CD8 T cells in a cross-sectional study involving HIV-2 and HIV-1 cohorts of patients paired for disease stage, as defined by CD4 counts. Also, thymic activity was estimated by the quantification of the signal joint/beta T cell receptor excision circle (sj/β TREC) ratio as described previously to indirectly assess intrathymic proliferation history (6-8). These data were correlated with the maintenance of peripheral CD4 T cell numbers in a cohort of untreated chronically HIV-2- and HIV-1-infected subjects.

In summary, the data presented here show that:

i) there is a correlation between circulating IL-7 levels and the degree of CD4 T cell depletion in HIV-2 infection, which is much stronger than the one found in the HIV-1 cohort. At the advanced stages of infection, similar levels of circulating IL-7 levels were found in both HIV-2 and HIV-1 cohorts, which were significantly higher than in the healthy cohort. However, in contrast to HIV-1 infection, the levels of circulating IL-7 were not significantly increased in the early stages of HIV-2 infection (9).

ii) the expression of the IL-7Rα was better preserved in all subsets of CD8 T cells and in memory CD4 T cells in HIV-2 in comparison with HIV-1 cohort, although there was a significant reduction in both cohorts as compared to the healthy controls. Moreover, a clear preservation of IL-7Rα expression in the naive CD4 T cells was shown in the HIV-2 cohort, with levels comparable to those found in healthy controls that did not occur in HIV-1 infected patients. (9).

iii) HIV-2 infected patients presented with elevated sj/β TREC ratios, which were compatible with a maintained thymic production for prolonged periods of time. Of note, in the healthy controls after 45 years of age there was a reduction in the sj/β TREC ratios in agreement with the expected thymic involution. However, in the HIV-2 infected cohort the sj/β TREC ratios were maintained independent of age (10).

Taken together, these data suggests that in HIV-2 infection, both thymopoiesis and the peripheral T cell homeostasis may be better preserved.

IL-7 in HIV/AIDS Pathogenesis

IL-7 is an essential cytokine for T cell homeostasis. In lymphopenic states, circulating IL-7 levels increase and during HIV-1 infection these levels have been show to correlate with the depletion of the CD4 T cell population (11, 12). In this work, it was showed for the first time, a strong inverse correlation between circulating IL-7 levels and the degree of CD4 T cell depletion in HIV-2 infection (9).

In the periphery, the mechanisms that drive increased circulating IL-7 levels remain unclear (13, 14). As it was previously proposed, elevated circulating IL-7 levels could result from an increased homeostatic response to the CD4 lymphopenia (11, 13, 15). On the other hand, the circulating IL-7 levels may increase as a result of its diminished binding and internalization by a reduction in the number of cells expressing the IL-7Rα (14, 16). The findings described here showing that in early stages of HIV-2 infection, the levels of circulating IL-7 were not significantly increased as compared to healthy cohort, and a much better preservation of the levels of IL-7Rα expression in the naive CD4 T cells than in HIV-1 infection, support the latter possibility. Thus, the delayed increase in circulating IL-7 levels throughout HIV-2 disease may result from the sustained expression of IL-7Rα, which suggests a maximization of available resources to allow the survival of the naive CD4 T cell population.

Interestingly, in early HIV-2 infection, circulating IL-7 levels were not only lower that in HIV-1 but also similar to the healthy cohort. To better interpret these results it would be relevant to understand how the HIV-2 infection is established. There are no clinical reports of HIV-2 acute infection and no available data on lymph node or mucosal pathology during HIV-2 disease. However, it is reasonable to speculate that the establishment of HIV-2 infection may not be associated with a high viremia peak and with a major depletion of the memory compartment. Recent data on acute HIV-1 infection, show a marked depletion of CD4 T cell subsets mainly in the gut, that persist throughout disease (17-19). It is plausible that the early depletion of this cell population significantly contributes to the higher circulating IL-7 levels. The possible contribution of gut-associated CD4 depletion in triggering the early increase of IL-7 production in HIV-1 infection that was not observed in HIV-2 infection deserves further exploration.

IL-7 is a key cytokine in T cell homeostasis in the periphery by promoting T cell proliferation and survival, particularly of the naive CD4 and CD8 T cell pools (12, 13, 20- 23). In HIV-1 infected patients, IL-7 was shown to mediate survival and expansion of naive and memory T cell populations and inhibits apoptosis of CD4 and CD8 T cells *in vitro* (24).
Recently, our lab showed that IL-7 preferentially induces proliferation of the $CD31^+$ naive $CD4^+$ T cell subpopulation in both adult peripheral blood cells and cord blood cells, with the maintenance of CD31 expression *in vitro* (25). The $CD31^+$ subset of naive CD4 T cells is

thought to be enriched in recent thymic emigrants (RTE) as described in the introduction of this work (26). Therefore, these data suggests that RTE in the naive T cell subset are more likely to respond to IL-7 by increasing their rate of homeostatic proliferation and still preserve RTE phenotype.

IL-7 therapy has been suggested to be a good candidate for inducing immune reconstitution in lymphopenic diseases. In T cell lymphopenic murine models, exogenous IL-7 administration or IL-7 transgenic over expression results in marked increases of naive CD4 and CD8 T cell numbers (27-29). In non-human primates exogenous IL-7 induces the expansion of naive peripheral T cells and enhanced *de novo* thymic production in SIV-infected animals (30-32). Several phase I/IIa clinical trials designed to assess the safety of administration of recombinant human IL-7 (rhIL-7) to humans, show absent or very low significant drug toxicity (33-35). Phase I studies of rhIL-7 therapy for HIV-1 infected patients showed a sustained increase in the naive CD4 and CD8 subsets (33, 34) with a transient expansion of RTE defined as $CD4^{+}CD45RA^{+}CD31^{hi}$ (34). Also, phase I studies of administration of rhIL-7 in patients with refractory cancer, showed a sustained increase in peripheral blood CD4 and CD8 T cells with a preferential expansion of RTE, and a broadening of TCR repertoire diversity measured by spectratyping (35).

These results suggest that rhIL-7 therapy, in combination with efficient antiretroviral therapy (ART), can increase the naive T cell pool, by enhancing peripheral proliferation with maintenance of naive phenotype. Additionally, a possible contribution of rhIL-7 therapy in the enhancement of thymopoiesis should be considered, as Beq et al showed for SIV infected macaques treated with IL-7 (30). Most likely it is a combined effect of enhanced thymopoiesis and naive peripheral expansion. Thus, these data suggested that rhIL-7 therapy could enhance and broaden immune responses, particularly in patients with limited naive T cells and diminished TCR repertoire diversity.

Importantly, rhIL-7 therapy showed a sustained increase in the central memory CD4 and CD8 T cell subsets with a transient increase in cell cycling and activation markers (33, 34). These expanded CD4 and CD8 T cells responded in vitro to TCR stimulation by proliferating and producing intracellular cytokines (34). Of note, exogenous IL-7 augments effector and memory responses to vaccination in animal studies and IL-7 therapy augments antitumor responses, leading to improved survival when combined with antitumor vaccines (36, 37).

On the other hand, IL-7 is a strong inducer of HIV-1 replication *in vitro* (38-40), leading to its proposed use as a therapy to purge the viral reservoirs in HIV-1 infected patients on ART with evidence of sustained suppression of circulating viral load (38). Importantly, in both communications of rhIL7 administration to HIV-1 infected patients with ART suppressed viral load, there were no described cases of sustained elevation of the viremia during rhIL-7 therapy (33, 34). However, 4 patients treated with higher dosages of rhIL-7 presented "blips" or intermittent elevations of the viral load that returned to basal levels (34). Of note, none of these studies were designed to address the impact of rhIL-7 therapy on the viral reservoirs on quiescent T cells. Other reports of SIV infected macaques treated with IL-7 also showed no increase in viral load (41). Thus, more data is necessary to really evaluate the effect of rhIL-7 therapy in viral purging, including the possible dosage adjustments required to achieve an effective reduction in the pool of latently infected cells.

Although there are no data on the ability of IL-7 to promote HIV-2 replication, it would be expected to be comparable given the similarity of the two viral promoter regions (LTRs) (42). It would be very important to clarify this issue given the low to undetectable HIV-2 viremia documented, in spite of the presence of HIV-2 proviral DNA levels in the range of those found in HIV-1 infection (43-45) and the high circulating IL-7 levels in advanced disease stages (9).

The Thymus in HIV/AIDS Pathogenesis

The impact of a functional thymus on HIV progression has not been fully established in part due to the difficulty in assessing thymic function or lack thereof, directly in infected patients.

Importantly, Dion et al proposed a marker for measuring thymic function, by estimating the intrathymic proliferation history of peripheral lymphocytes based on the quantification of the sj/β TREC ratio (6, 8, 46). Using this technique Dion *et al* (6), showed suppression of intrathymic proliferation in HIV-1 infected patients in the initial phase of disease. The data presented here showed for the first time that HIV-2 infected patients have elevated sj/β TREC ratios, suggesting a maintained thymic production throughout HIV-2 disease. Moreover, in HIV-2 infected patients, the sj/β TREC ratios were maintained independent of age as opposed

to the healthy controls that presented an age dependent reduction in the sj/β TREC ratios, in parallel with the expected thymic involution (10).

Thus, the data presented are compatible with a thymic preservation or rebound in HIV-2 infected patients, possibly contributing to the sustained replenishment of the naive T cell pool and to the slower rate of CD4 decline that distinguishes HIV-2 from HIV-1 infection. In agreement, in HIV-1 infected patients with a slower rate of disease progression, it was demonstrated that the maintenance of circulating CD4 T cells is strongly associated with efficient thymopoiesis (46).

However, there are no data on the direct impact of HIV2 infection on the thymus. It would be important to further investigate the impact of HIV-2 infection on the thymus, in particular to determine if the different thymocyte populations are permissive to HIV-2 infection and replication. Furthermore, it would relevant to investigate the impact of HIV-2 on T cell development and on $CD34^+$ progenitor differentiation, which have been shown to be compromised during HIV-1 infection (47).

The maintenance of thymic activity and naive T cell homeostasis is considered fundamental for the development and preservation of a broadly diverse T cell repertoire in the periphery. This is thought to be critical for the organism to maintain the ability to mount responses to new pathogens, prevent relapsing of latent microorganisms and delay disease progression in persistent infections.

Thus, in order to study thymic function it is of great importance to quantify the diversity of the TCR repertoire. This has not been formally addressed due to limitations of the methodologies currently available to measure diversity. It would be very relevant to find a good reliable technique that measures TCR repertoire diversity in order to have a reliable measurement of thymic function.

The current mostly used approach devised to do so, immunoscope or spectratyping, is based on the fact that during rearrangement of the α and β regions of the TCR, nucleotides are incorporated to increase diversity of segments between the VDJ regions. Given that this is a random process, the quantification of the nucleotides incorporated in the CDR3 regions in all

the T cells should follow a Gaussian curve, as reported in samples from healthy individuals. In the presence of a skewed repertoire, due to manipulation or disease, the curves of the CDR3 are non-Gaussian in shape, since not all the possible CDR3 lengths exist in a contracted repertoire (48). However in the context of HIV pathology this methodology should be used with caution, due to the possible oligoclonal expansions associated with T cell responses to antigens, both from HIV or other opportunistic infections. Thus, the skewing of the Gaussian curve in a sample of T cells from an HIV infected patient may not be related to the disruption of thymic function but to clonal expansions and relative imbalances of T cell subpopulations represented in the assessed sample.

Therefore, a reliable method that quantifies the diversity of the TCR repertoire with adequate sensitivity to detect and identify minor changes and/or clonal expansions is still critically necessary, despite promising recent approaches (49). Such an assay could be very important not only to the understanding of HIV/AIDS pathology but also for the evaluation of immunodeficiencies and immune reconstitution in general.

Final remarks

In conclusion, the overall objective of this work was to contribute to the better understanding of mechanisms and implications of HIV mediated loss of thymic function and impaired T cell homeostasis, through the use of an attenuated natural model of HIV disease, the HIV-2 infection.

The data discussed here point to a better maintenance of thymopoiesis and peripheral use of IL-7 during HIV-2 infection. Devising ways to improve these parameters in HIV-1 infected patients in conjunction with ART may contribute to a better outcome and an improvement in the quality of life of the people affected by this disease.

HIV1 infection has been under study for the past 26 years and every piece of evidence is a small stone in the path of understanding and finding effective therapy options and prophylactic approaches.

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PART II

TACI REGULATION OF B CELL DIFFERENTIATION AFTER ANTIGENIC STIMULATION

"I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale." **Marie Curie**

Introduction

Deficiencies in the Transmembrane Activator and Calcium Modulating Cyclophilin Ligand Interactor (TACI) receptor are associated with Common Variable Immunodeficiency (CVID) in humans (1, 2). CVID is a primary immunodeficiency that comprises a heterogeneous group of diseases with deficient immunoglobulin (Ig) production (3-6). It is the most prevalent human primary immunodeficiency with clinical expression, affecting 1 in 25,000 Caucasians (7, 8); 5% to 10% of subjects with CVID carry at least one germline mutation in the TACI *locus* (1, 2, 9).

TACI deficiency causes recurrent bacterial infections, particularly of the respiratory tract (1, 2, 10) and impaired responses to vaccination against encapsulated bacteria such as *Streptococcus pneumoniae* or *Haemophilus influenzae* (2). The impaired clearance of encapsulated bacteria in TACI deficient subjects has been attributed to reduced IgG and IgA production owing to a B cell defect. In support of a primary B cell defect, Salzer *et al* (1) showed that some TACI deficient subjects have decreased number of $CD27^+$ antigen experienced B cells. How TACI promotes antibody production is not understood and the subject of the second part of this work.

TACI deficiency in humans and in mice

Recently, mutations in the human TACI locus were found in 5% to 10% of patients diagnosed with Common Variable Immunodeficiency (CVID) (1, 2). Individuals with CVID exhibit deficient production of antibodies of all isotypes (1, 2). Because most CVID subjects have relatively normal numbers of B lymphocytes, defective Ig production is thought to reflect defective activation and differentiation of B cells. (3, 11). Consistent with this idea, plasma and memory B cells are reduced (8, 12-14). Also, individuals with CVID suffer from recurrent otitis and sino-pulmonary infections typically due to encapsulated bacteria such as *Haemophilus influenza* and *Streptococcus pneumoniae* (5, 6). CVID also causes increased incidence of autoimmune disorders and of lymphoid and nonlymphoid malignancies (8, 12, 15).

Several point mutations in the TACI *locus*, *TNFRSF13B*, have been described and are depicted in *Figure 1*. The most commonly found, C104R or A181E, do not abolish expression; in contrast, S144X, S194X and an insertion, 204insA, result in decreased or abolished expression (16, 9, 17). In one recent study (16) Salzer et al found that all patients with biallelic mutations had hypogammaglobulinemia. In another study, patients with monoallelic changes in *TNFRSF13B* and heterozygosity for the most common TACI mutation, C104R, had antibody deficiency, suggesting that some mutated TACI proteins exert dominant negative functions (9, 17).

TACI deficient mice mount deficient IgG antibody responses to polysaccharides and have decreased serum IgA and IgG (18). Defective antibody responses by TACI-deficient mice were not owed to decreased B cells since TACI-deficient mice have more B cells than wild-type mice, including more marginal zone B cells ($IgM^+IgD^-CD21^+$) and B1 B cells ($CD5^+IgM^+$ B cells, present mainly in the peritoneal cavity) which are thought to contribute most of the antibodies to polysaccharides. Thus, TACI deficiency does not appear to impair the development or maintenance of polysaccharide-responsive B cells in mice (18). In fact, Yan *et al*. (19) showed that TACI deficient B cells hyperproliferate in response to lipopolysaccharides (LPS) and that TACI deficient mice develop splenomegaly and enlarged lymph nodes (18), suggesting that TACI may inhibit B cell proliferation. Thus TACI deficiency may on one hand be a B cell co-receptor necessary to evoke anti-polysaccharide

responses (18), and on the other hand inhibit B cell proliferation (19, 20). How TACI combines inhibitory and stimulatory B cell functions is not understood.

There are several differences in the phenotypes associated with TACI deficiency in humans and in mice. While TACI deficient mice have increase number of B cells, human subjects with TACI deficiency have normal or slightly reduced number of B cells including decreased $CD27^+$ memory B cells in the blood of most patients (1, 16). In TACI deficient mice there is a reduction mostly in IgG and IgA subclasses while in human subjects TACI deficiency all isotypes are reduced (2). Both mice and human subjects with TACI deficiency have impaired responses to vaccination against *Pneumococcus* indicating defective antibody production to polysaccharide antigens (2). While TACI deficient mice develop systemic lupus erythematosus, TACI deficient subjects develop mostly other autoimmune diseases such as Graves disease, pernicious anemia, rheumatoid arthritis, Sjogren's, hemolytic anemia with idiopathic thrombocytopenic purpura (5). Autoimmune manifestations may not be a direct cause of TACI deficiency since these manifestations also occur in individuals with CVID due to mutations in genes other than TACI (21).

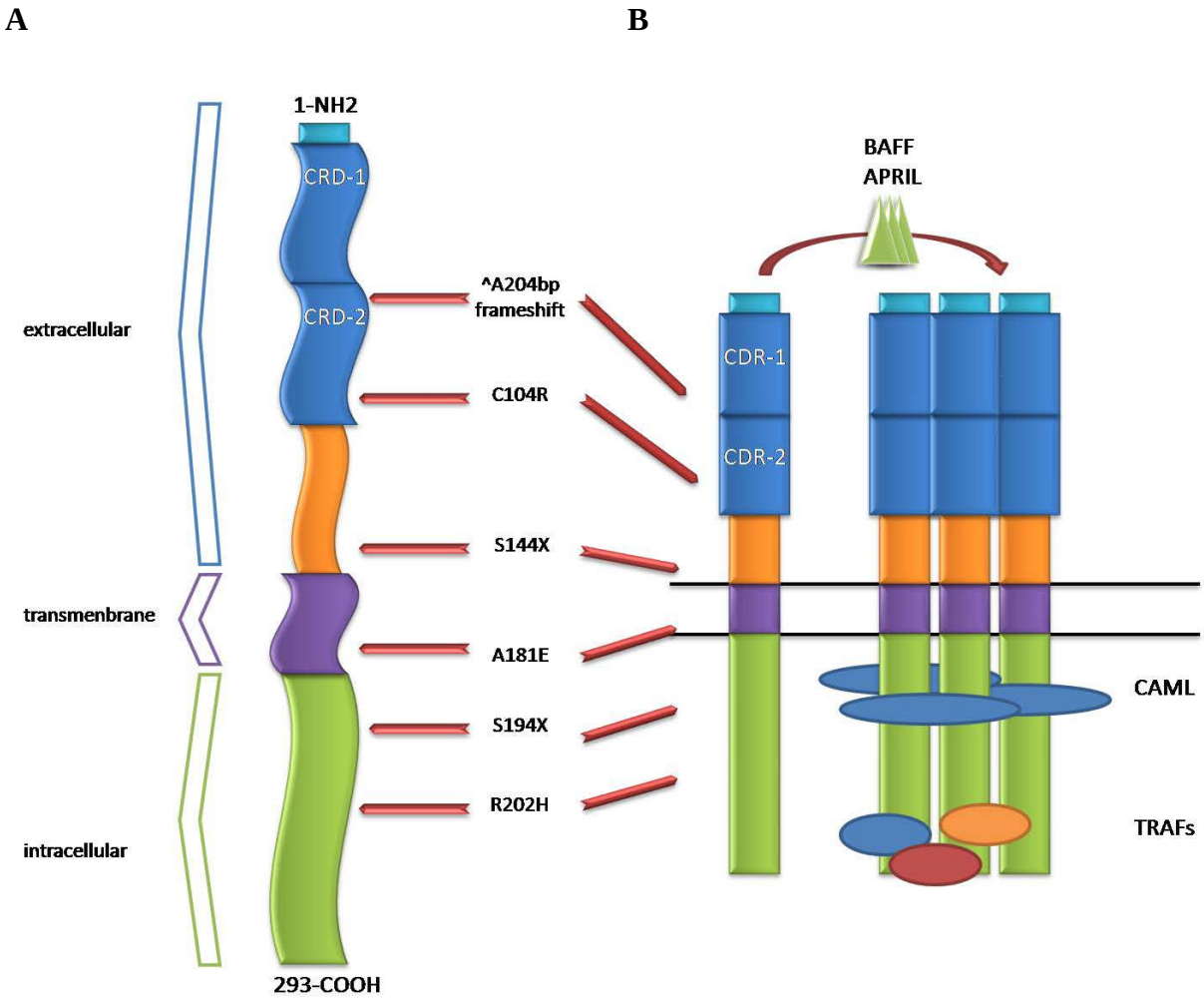

Figure 1 – (A) Summary of mutations in the TACI locus, TNFRSF13B, that have been described to date in CVID patients. (B) Schematic representation of TACI structure and ligand dependent recruitment of intracellular signaling (CAML, TRAFs) by TACI ligands (APRIL, BAFF).
Adapted from Castigli, *et al* (17).

TACI intracellular signaling and ligand interactions

TACI is a type III transmembrane protein receptor, with a structure typical of the tumor necrosis factor (TNF) receptor superfamily (Reviewed in (22, 23)). In humans TACI is expressed by marginal zone B cells and by $CD27^+$ memory B cells (24, 25) and TACI expression is enhanced after B cell stimulation (26), whereas murine TACI is constitutively expressed by all B cells and activated T cells (24, 27). B cells produce two TACI alternative splicing variants with extra-cellular N-terminal domains with one or two cysteine-rich domains (CRDs) that include a conserved six-residue motif, (F/Y/W)-D-x-L-(V/T)-(R/G) required for ligand binding and receptor activation (28, 29).

TACI has two known TNF-like ligands that bind with high affinity, B cell activating factor (BAFF, also known as BLyS) (30-33) and a proliferation inducing ligand (APRIL) (34-36). APRIL and BAFF share several features, both are synthesized as type II transmembrane proteins with a TNF homology C terminal domain and are proteolytically cleaved into soluble form at a multibasic motif by a furin-like protease (37); both are expressed in monocytes-macrophages and dendritic cells (38) and BAFF is also expressed by neutrophils (39). BAFF and APRIL also bind to B cell maturation antigen (BCMA) receptor (35) and BAFF binds to B cell activating factor receptor (BAFF-R) (40). TACI is the only receptor that binds BAFF/APRIL heterotrimers (28, 41). Bischof and collaborators (42) showed that TACI also binds to syndecan-1, -2 and -4, membrane-bound proteoglycans that contain large extracellular domains with heparan-sulfate post-translational modifications. In contrast to BAFF and APRIL, syndecans bind only TACI and not BAFF-R or BCMA as illustrated in *Figure 2* (42).

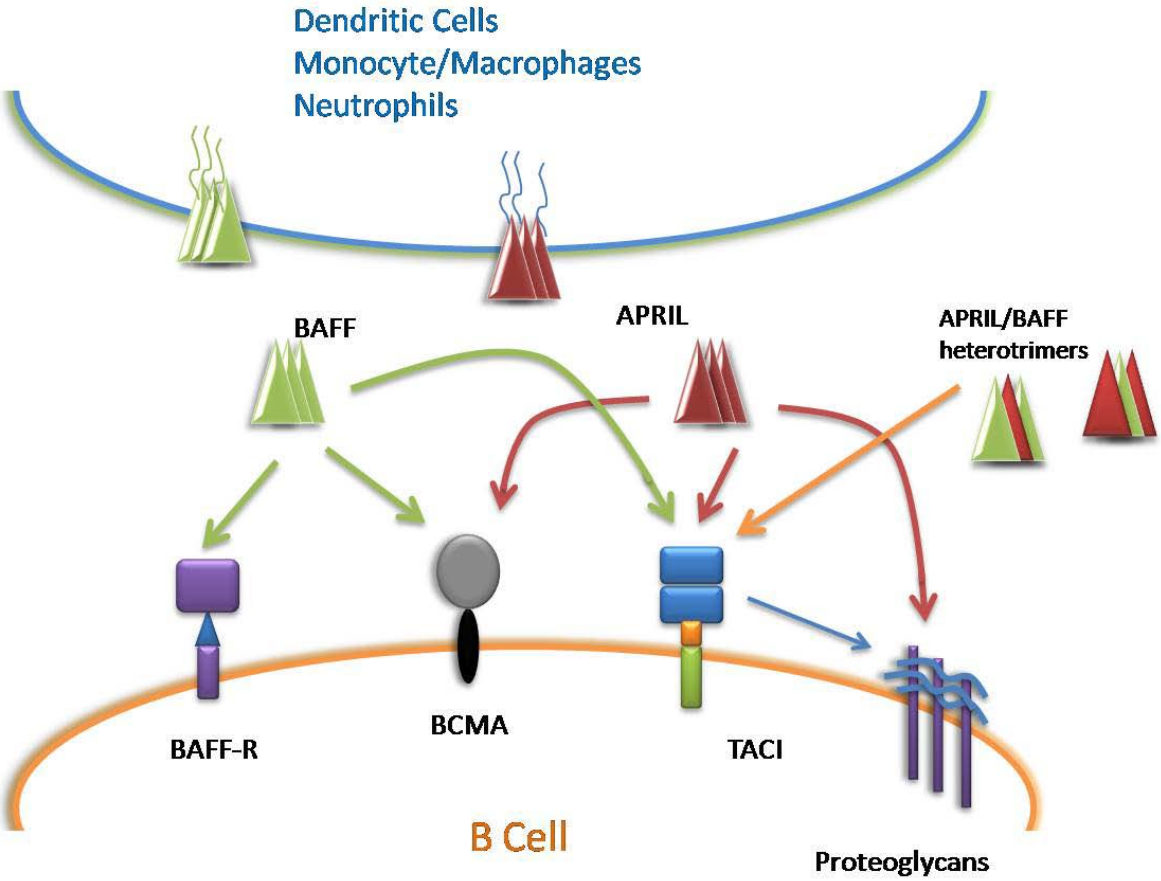

Figure 2 – Representation of TACI and its ligands, BAFF and APRIL and their receptors, BCMA and BAFF-R.

TACI is the only receptor that binds BAFF/APRIL heterotrimers (28, 41). APRIL and TACI also bind to membrane-bound proteoglycans that contain large extracellular domains with heparan-sulfate post-translational modifications. (42). Adapted from Castigli, *et al* (43).

TACI receptor activation initiates two signal transduction pathways. In one, TACI recruits TNF receptor associated factors (TRAF) 2, 5 and 6 (44), and in another TACI binds the calcium modulating cyclophilin ligand (CAML) as depicted in *Figure 3* (27).

TRAF 2, 5 and 6 activate nuclear factor kappa B (NF-κB) (44, 45) and c-Jun NH2-terminal kinase (JNK). Activation of NF-κB and JNK control many steps in the differentiation of B cells, including proliferation, class switch recombination and plasma cell differentiation (46-48) but because these pathways can be activated by many different receptors their function does not uniquely reflect activation of TACI.

TACI binds to CAML (27), and the TACI-CAML binding activates phospholipase C-γ (PLC-γ), which hydrolyses phosphatidylinositol-4,5-bisphosphate to produce inositol-1,4,5-trisphosphate (InsP3) and diacylglycerol. InsP3 induces the release of calcium from intracellular stores. Calcium binds calmodulin, which in turn activates the calmodulin-dependent phosphatase, calcineurin (49). Nuclear factor of activated T cells (NFAT) proteins are then dephosphorylated by activated calcineurin, which leads to their nuclear translocation and the induction of NFAT-mediated gene transcription as recently reviewed by F. Macian (50). TACI-CAML interactions promote translocation of the transcription NFAT to the cell nucleus, because NFAT translocation is specifically blocked by a dominant-negative CAML mutant (27, 51).

In the nucleus, NFAT interacts with activator protein 1 (AP-1) to promote transcription of genes that encode transcription factors, signaling proteins, cytokines, cell surface receptors, and other effector proteins, as reviewed by Rao *et al* (52). In B cells, AP-1 represses B cell lymphoma 6 (BCL-6), a transcriptional repressor protein (53, 54). Repression of BCL-6 is necessary to drive differentiation of plasma cells through the expression of B lymphocyte-induced maturation protein-1 (Blimp-1) (55). It is possible that TACI deficient B cells differentiate poorly into plasma cells owing to incomplete activation of AP-1.

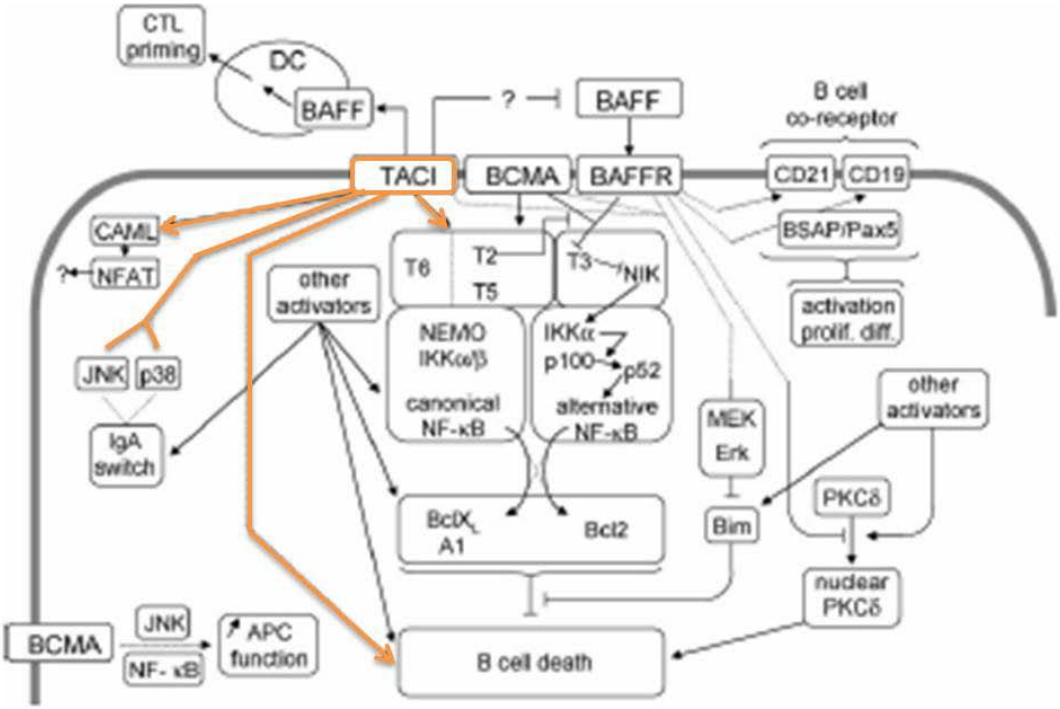

Figure 3 – Schematic representation of signaling pathways downstream of BAFF receptors.
The molecular events that lead to B cell survival, Ig swich or other responses downstream of BAFF-R, TACI (highlighted) or BCMA are poorly characterized. Adapted from Bossen, *et al* (22).

The phenotype caused by TACI deficiency may be owed to the lack of receptor function but also to the compensatory changes associated with the increased availability of ligands to bind the BAFF-R and BCMA. Thus, defective antibody responses to polysaccharides in TACI deficient mice are not due to deficient APRIL signaling since APRIL-deficient mice have normal antibody responses to polysaccharides (56). However over-expression of APRIL enhances antibody responses to polysaccharides (57) presumably by activating TACI since BCMA is dispensable for antibody responses to polysaccharides (58). BCMA promotes antibody production in response to proteins possibly by enhancing plasma cell survival (59). IgA production may require the binding of APRIL to TACI (60) with the exclusion of other ligands or receptors because APRIL deficient mice, as TACI deficient mice, show a selective deficiency of IgA (61).

TACI deficiency increases the B cell compartment (18-20) which is consistent with an increased availability of BAFF to bind the BAFF-R. Administration of BAFF to mice increased the number of B cells (62) and conversely, lack of BAFF in mice severely contracted the B cell compartment and reduced antibody responses (63, 64) indicating that BAFF controls B cell homeostasis.

Aim

TACI appears to have both inhibitory and activating roles in B cell differentiation; on one hand, TACI inhibits uncontrolled B cell proliferation; on the other promotes B cell differentiation and Ig isotype class switch mostly in response to polysaccharide antigens. How TACI accomplishes such distinct responses was the main objective of this work.

To determine the origin of the antibody deficiency caused by TACI deficiency, TACI KO mice were bred with Quasi-Monoclonal (QM) mice that are genetically engineered to produce a contracted antibody repertoire. The Quasi-Monoclonal mouse (QM) was engineered by Cascalho *et al* (65) as a model for studying the generation of antibody diversity. In QM mice, 80% of the peripheral B cells express a BCR encoded by a knock-in VH17.2.25 heavy chain and the λ1 or λ2 L chain that binds to a hapten, 4-hydroxy, 3-nitro-phenylacetic acid (NP) and its derivatives. These mice mount enhanced responses to polysaccharide-like antigens such as NP conjugated to Ficoll owing to increased antigen-specific marginal zone B cells (IgM^+ IgD^- $CD21^+$) (66). Marginal zone B cells are thought to constitute the first line of response to encapsulated microbes.

Thus, QM TACI KO mice immunized with NP-Ficoll, a polysaccharide like T-independent antigen, were studied, the frequencies of antibody secreting B cells were measured and the amount of antibodies produced was quantified, in order to clarify the mechanism by which TACI controls antibody responses to polysaccharides.

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Results

TACI IS REQUIRED FOR EFFICIENT PLASMA CELL DIFFERENTIATION IN RESPONSE TO T-INDEPENDENT TYPE 2 ANTIGENS.

Publication:

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GTM and CSC contributed equally to this work.

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Abstract

The control of systemic infection by encapsulated microorganisms requires T-independent type II (TI-2) antibody (Ab) responses to bacterial polysaccharides. To understand how such responses evolve, we explored the function of transmembrane activator calcium modulator and cyclophilin ligand interactor (TACI), a member of the TNFR family, required for TI-2 Ab production. Quasimonoclonal (QM) mice produce robust TI-2 responses to 4-hydroxy-3-nitrophenylacetate (NP)-Ficoll, owing to the high precursor frequency of NP-specific B cells in the marginal zone of the spleen. QM mice that lack TACI produce decreased numbers of IgM (2-fold) and IgG (1.6-fold) NP-specific antibody-secreting cells (ASCs) compared with TACI-positive QM mice in response to immunization with NP-Ficoll. Our studies indicate that TACI acts at a remote time from activation because TACI is not necessary for activation and proliferation of B cells both in vitro and in vivo. Instead, TACI-deficient QM B cells remained in the cell cycle longer than TACI proficient QM cells and had impaired plasma cell differentiation in response to NP-Ficoll. We conclude that TACI has dual B cell-autonomous functions, inhibiting prolonged B cell proliferation and stimulating plasma cell differentiation, thus resolving the longstanding paradox that TACI may have both B cell-inhibitory and -stimulatory functions. By promoting plasma cell differentiation earlier during clonal expansion, TACI may decrease the chances of autoantibody production by somatic hypermutation of immunoglobulin (Ig) genes in response to T-independent antigens (Ags).

Introduction

T-independent type II antigens (TI-2 Ags) are large polysaccharides found in the capsules of such bacteria as *Streptococcus pneumoniae, Haemophilus influenza* and *Klebsiella pneumoniae.* These capsular polysaccharides do not activate the lectin or alternate pathways of complement; hence, activation of the classical pathway by antibodies (Abs) is required for effective opsonization (1). Humans with impaired ability to mount Ab responses to polysaccharides, including young children and splenectomized adults, are at increased risk of life-threatening infections from encapsulated bacteria (2).

To study Ab responses to polysaccharides we took advantage of the quasi-monoclonal (QM) mice. In the QM mice 80% of the peripheral B cells express a BCR encoded by a knock-in VH17.2.25 heavy (H) chain and the λ1 or λ2 light (L) chain (3). These mice mount enhanced responses to 4-hydroxy-3-nitrophenylacetate (NP)-Ficoll (a TI-2 Ag), owing to an increased B cell precursor frequency with a marginal zone phenotype (IgM-positive, IgD-negative and CD21-positive) (4). The marginal zone B cells are thought to constitute the first line of response to encapsulated microbes (5).

What special properties of B cells besides the B cell receptor (BCR) may allow TI-2 responses to occur is not known. We explored the functions of the trans-membrane activator calcium modulator and cyclophylin ligand interactor (TACI), found previously to be needed to mount effective TI-2 Ab responses (6). TACI has two known ligands: B lymphocyte stimulator (BLyS) and the proliferation inducing ligand (APRIL). Administration of BLyS to mice increased B cell numbers and enhanced Ab responses to both T-dependent Ags and TI-2 Ags (7). Conversely, mice that lack BLyS had severely reduced B cell numbers and reduced Ab responses (8). In contrast, APRIL knockout (KO) mice have normal TI-2 responses (9), but transgenic expression of APRIL led to increased TI-2 Ab responses (10).

BLyS and APRIL bind to two other receptors B cell activating factor receptor (BAFF-R) and B cell maturation Ag (BCMA) in addition to TACI. BCMA promoted plasma cell survival in T-dependent responses (11) but was dispensable for TI-2 Ab responses (12); mutations in the intracellular domain of BAFF-R (A/WySnJ mice) caused reduced Ab responses to both TI-2 Ags and T-dependent Ags and decreased numbers of peripheral B cells (13). Thus,

contribution of TACI for TI-2 Ab responses in response to BLyS and APRIL remains unclear. Previous work by the authors revealed that TACI binds uniquely to heparan sulfate post-translational modifications of syndecan-2 (14). Thus, some of the unique functions of TACI may be attributed to binding of TACI to heparan sulfate on syndecan-2.

Mice lacking TACI are unable to mount effective TI-2 Ab responses (6), suggesting that TACI is needed for the generation of these responses. How TACI promotes these responses is not known. TACI-KO mice have increased numbers of all B cell subtypes (6), including marginal zone B cells (15) and B1 B cells (16), suggesting that TACI deficiency does not impair the development of polysaccharide-responsive B cells. This contrasts with models with defective TI-2 Ab responses, owing to decreased or absent B cells in the marginal zone of the spleen or in the serosal cavities (B1 B cells). In contrast to the apparent impact on TI-2 responses, TACI is thought to inhibit some B cell functions. Thus, TACI-/- B cells hyper-proliferate in response to polyclonal activators *in vitro*, and loss of TACI causes autoimmunity in mice (6, 17, 18), suggesting that TACI is a B cell inhibitory receptor. How TACI combines inhibitory and stimulatory B cell functions is not understood. Here we asked whether TACI regulates B cell differentiation following T-independent stimulation of B cells.

To determine the mechanism by which TACI controls TI-2 Ab responses, we bred TACI-KO mice with the QM mouse (3) to obtain TACI-negative QM mice. Because in QM mice the transgenic heavy chain was targeted into the JH locus, it can undergo isotype switching and somatic hypermutation. We studied how TACI promotes QM B cell responses to NP-Ficoll (a TI-2 Ag). We found that QM B cells lacking TACI are normally activated by Ag but proliferate longer and have impaired plasma cell differentiation. Our findings suggest that TACI acts remotely from B cell activation and provides a critical signal for cells to exit the cell cycle and to differentiate into plasma cells.

Materials and Methods

Mice and Immunizations:

TACI-KO and QM mice were previously described (3, 6). TACI-KO mice were bred to QM mice and are in a C57BL/6 (B6) background. Animals were bred and treated according to the policies of Mayo Foundation Institutional Animal Care and Use Committee. Mice were immunized with 30 μg intra-peritoneally (i.p.) of NP-Ficoll (NP41-AECM-Ficoll, Biosearch technologies) diluted in sterile PBS.

Isolation of primary B cells:

$CD19^+$ splenocytes were isolated by negative selection with a B cell isolation kit (Miltenyi Biotec), as per the manufacturer's protocol. Cells were grown in RPMI-1640, 10%FCS, 1%v/v Pen/Strep, 55 μM 2-ME.

ELISA:

NP-specific Abs in the sera were determined using ELISA, as previously described (19).

ELISPOT:

Multiscreen HTS-HA 96 well plates purchased from Millipore were coated with 5 μg/ml NP-BSA or 5 μg/ml BSA in sodium carbonate buffer overnight at 4^oC. Plates were blocked with 5% milk in TBS-Tween, and B cells obtained by negative-selection with magnetic miltenyi microbeads (MACS) were serially diluted from a maximum of $2x10^5$ or $5x10^5$ cells per well, and cultured overnight. ELISPOTS from splenocytes isolated from adoptively transferred mice were not further processed because recipient mice lack T cells and were transferred with isolated B cells. Ab secreting cells (ASCs) were detected with AP-conjugated goat anti-mouse IgM or IgG Ab (Southern Biotech) for 2 hrs at 37^oC. The plates were developed with 5-bromo-4-chloro-3-indolyl phosphate-NBT substrate (SigmaFast, Sigma Co), and dots counted by microscopy.

Thymidine incorporation:

Splenocytes or negatively selected B cells isolated using MACS B cell isolation kit (Milteny Biotec) were plated at concentration $1x10^6$ cells /ml and stimulated with NP-Ficoll or LPS at the concentrations shown. At 72 h after the stimulation, 1 μCi [3H]TdR (MP Biomedicals)

per well was added for an additional 16-20 h. At the end of the culture period 50-60% of cells were viable as determined by trypan blue exclusion. Cells were lysed by hypotonic lysis, transferred to glass fiber filters (Packard) and counted on Matrix96-Direct Beta Counter (Packard).

Adoptive transfer:

Ten million negatively selected B cells isolated with the B cell isolation kit (Milteny Biotec) were injected into the jugular vein of RAG1-/- on B6 background 1 h before immunization.

FACS staining:

Biotinylated anti-idiotypic Ab (R2.438.8) directed against the QM Ab (17.2.25 monoclonal) was a generous gift from Dr. Imanishi-Kari (Tufts University). All other Abs were purchased from BD Pharmingen. FITC-conjugated mAbs were anti-mouse CD21 (7G6), IgDa (AMS9.1), GL7 and B220 (RA3-6B2). PE-conjugated mAbs were anti-mouse CD23 (B3B4), IgMa (DS-1), CD138 (281-2) and FAS (CD95). Biotynilated mAbs were anti-mouse IgDa (AMS9.1) and CD138 (281-2). Allophycocyanin-conjugated mAbs were anti-mouse CD19 (ID3) and B220 (RA3-6B2). Peanut aglutinin (PNA) was biotynilated (Vector Laboratories). Biotynilated Abs were revealed by streptavidin-PE-Cy5 purchased from BD Pharmingen. Data was collected using FACScan or FACSCalibur and analyzed with Cellquest software. For Annexin V binding assay, the cells were first stained with Abs directed to surface Ags and later stained with AnnexinV-PE Apoptosis Detection kit 1 (BD Pharmingen) according to the manufacturer's recommended protocol. Cell cycle analysis was done according to previously described procedures (20) in isolated B cells.

Results

TACI deficiency compromises IgG TI-2 Ab production and formation of ASCs in QM mice

To explore the involvement of TACI in TI-2 Ab responses, we bred TACI-KO mice(TACI-/-) with QM mice that have enhanced TI-2 responses to NP-Ficoll, in part owing to increased marginal zone NP-specific B cells (4). QM TACI-/- mice had 2.3-fold more splenocytes and B cells than QM TACI +/+ mice (Figure 1a). The increase in the number of B cells in QM TACI-/- mice did not alter the proportions of naive (IgM^+ IgD^+, 51% in TACI-/-versus 43% in TACI+/+), marginal zone($CD21^{+++}$, $CD23^+$, 42% in TACI-/- versus 44% in TACI+/+) or follicular($CD21^+$, $CD23^{+++}$, 31% in TACI-/- versus 32% in TACI+/+)B cells (Figure 1b).
QM mice respond vigorously to NP-Ficoll, a TI-2 stimulus, by producing NP-specific IgM (3246 μg/ml) and IgG3 (9.0 μg/ml) in the serum 4 days after immunization. QM mice deficient in TACI produced only 1.6 μg/ml NP-specific serum IgG3, 5.6-fold less than TACI-positive QM littermates, and comparable levels of NP-specific serum IgM (2673 μg/ml) indicating that TACI promotes IgG3 production in response to TI-2 stimuli in QM mice.

Next, we asked whether TACI was required to produce QM ASCs in the spleen following immunization with NP-Ficoll. The number of NP-specific ASCs was determined by ELISPOT 6 days after immunization. TACI-negative QM mice showed 2-fold decreased numbers of IgM and 1.6 fold-decreased numbers of IgG NP-specific ASC per spleen, compared to TACI-positive QM mice (Figure 2a). The number of IgM or IgG ASCs in the bone marrow was also decreased albeit not significantly, by 1.8- and 1.2-fold respectively, in TACI-negative mice compared to TACI-positive QM mice. Analysis of spleen sections showed that the number of B cells that differentiated into plasma cells ($CD138^+$) peaked at day 6 and slowly decreased thereafter (not shown). The number of plasma cells in the perifollicular areas of the spleen was significantly decreased in TACI-negative compared to TACI-positive QM mice 6 days after immunization (Figure 2b). Given that the precursor frequencies of NP-specific B cells were the same in TACI-negative QM and TACI-positive QM mice (approximately 85% for both, data not shown), our findings of reduced splenic ASC in QM mice deficient in TACI indicate that TACI promotes terminal differentiation. Decreased numbers of plasma cells were not due to increased apoptosis in TACI-negative QM mice, because TACI-negative $CD138^+$ cells had reduced apoptosis (Figures 3e and 3f), a

finding consistent with published reports indicating that an EDAR-TACI fusion induces death in the A20 mouse plasma cell line (17).

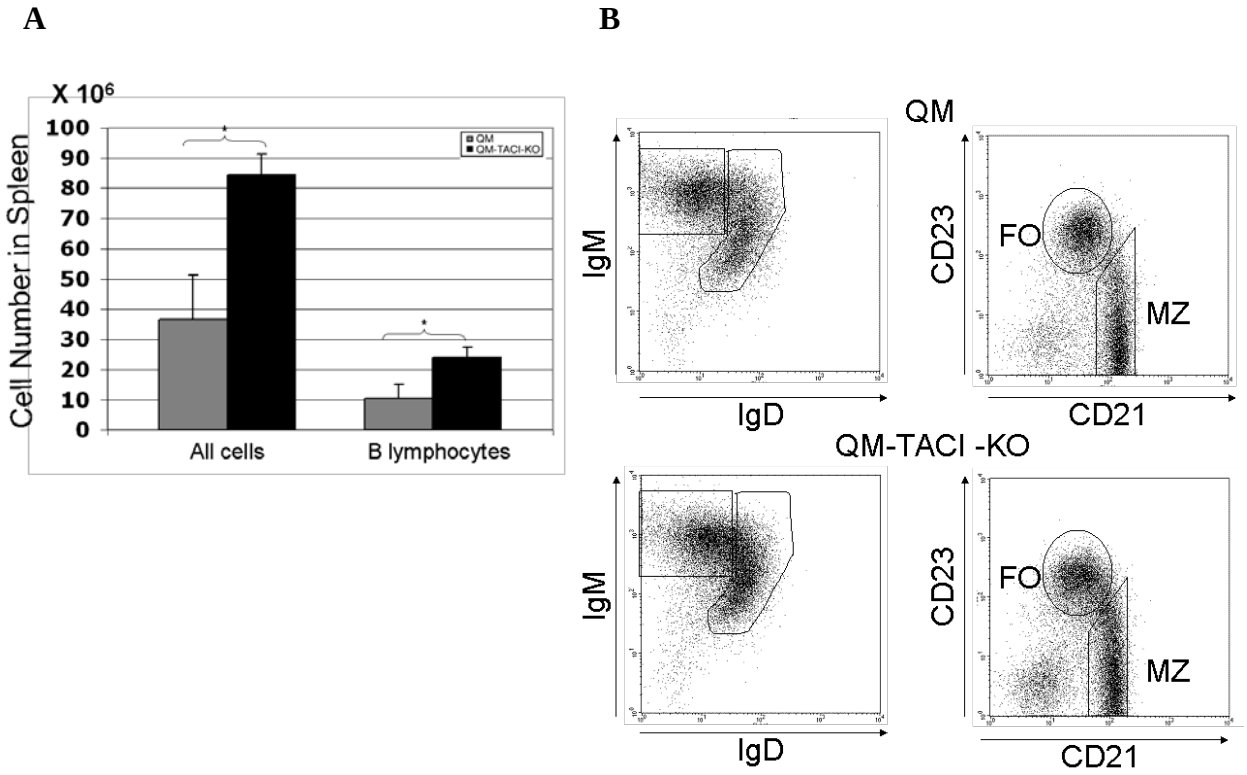
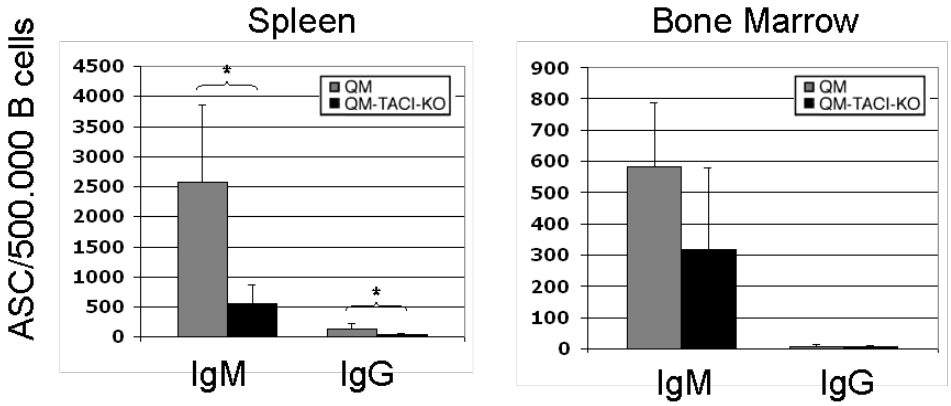


Figure 1. QM TACI-deficient mice have an increased number of B cells in the spleen and maintained marginal zone (MZ)/follicular B cell subset distribution.

Splenocytes from QM mice (QM, *n*=3) or QM-TACI^{-} (KO) littermates (*n*=3) were counted and analyzed by flow cytometry. **(A)** Numbers of splenocytes and CD19^{+} B cells in QM or QM-TACI^{-} mice (KO), as indicated. The number of B cells was calculated by multiplying the fraction of CD19^{+} B cells identified by FACS by the number of splenocytes. QM mice had, on average, 37+/-15 million splenocytes and 11+/-5 million B cells; QM-TACI^{-} mice had, on average, 84+/-7 million splenocytesand 24+/-3 million B cells. *, Significant differences. **(B)** Flow cytometry analysis of splenocytes. A typical plot is shown (from three independent experiments). Plots were obtained by gating on the lymphocyte gate defined on light scatter plots and on CD19^{+} cells. *y*-axis, fluorescence intensity of splenocytes stained with anti-mouse IgM or anti-mouse CD23 Abs PE-conjugated; *x*-axis, fluorescence intensity of splenocytes stained with anti-mouse IgD- or anti-mouse CD21 Abs FITC-conjugated, as indicated.

A



B

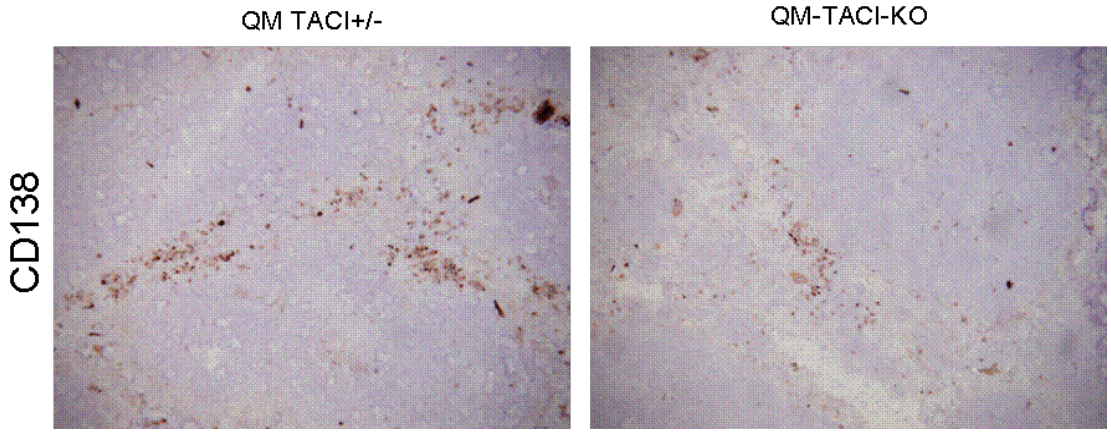


Figure 2. TACI deficiency leads to impaired antibody-secreting cell (ASC) formation after TI-2 immunization.

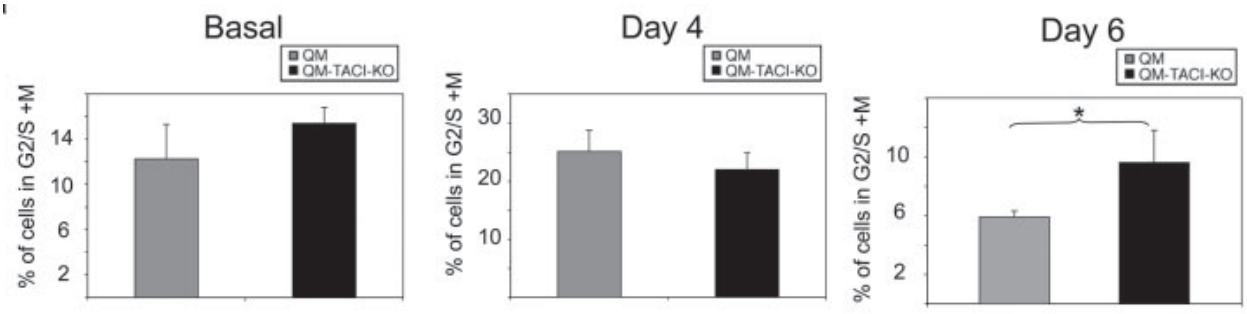
(A) The number of IgM or IgG ASCs was determined by NP-ELISPOT of B cells purified from the spleen or bone marrow 6 days after i.p. immunization of QM or QM-TACI-KO mice (*n*=5 mice per cohort) with 30 μg of NP-Ficoll. TACI^{-} mice had only 564 IgM or 33 IgG ASCs whereas TACI+ mice had 2579 IgM or 125 IgG ASC per 500,000 B cells from the spleen. After correction for the increased number of B cells in the spleen of TACI^{-} mice, our results indicate that TACI deficiency causes a 2-fold decrease in the absolute number of IgM-producing cells and 1.6-fold decrease in the number of IgG producing cells in the spleen, 6 days after immunization. TACI^{-} mice had 318 IgM or 6.3 IgG ASCs, whereas TACI+ mice had 584 IgM or 7.7 IgG ASC per 500,000 B cells in the bone marrow. *, Significant differences. **(B)** Spleen frozen sections obtained on day 6 after immunization, stained with anti-CD138 Abs to detect plasma cells. Image shows reduced accumulation of plasma cells in the perifollicular areas in TACI^{-} mice. Images are representative of images obtained from three independent experiments.

TACI deficiency causes B cells to remain proliferating longer and to differentiate less after TI-2 Ag stimulation in vivo

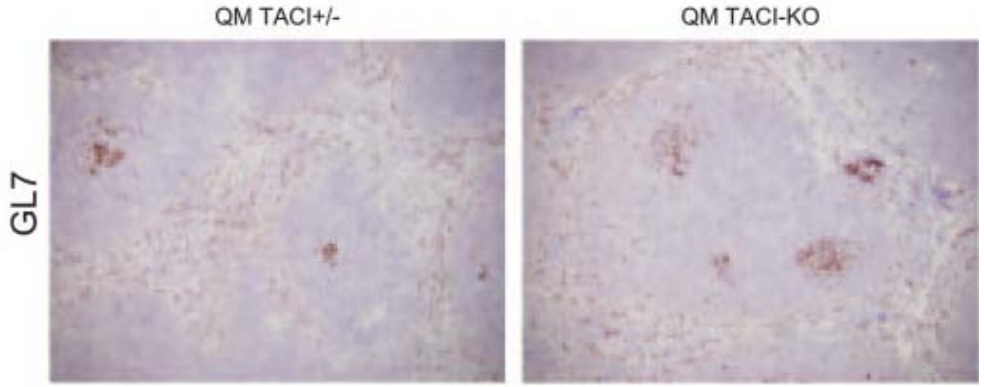
We next tested whether TACI promotes B cell differentiation early or late relative to B cell activation. We first determined if TACI deficiency enhances QM B cell proliferation by calculating the relative number of $CD19^+$ cells in the G_2-S and M stages of the cell cycle after immunization. Figure 3a shows that 6 days after immunization there were more TACI-negative QM B cells cycling (in G_2-S or M stages of the cell cycle, 9.6% on average), compared with TACI-positive B cells (5.9%, on average). Curiously, the number of TACI-positive and TACI-negative B cells cycling 4 days after immunization is similar, suggesting that TACI is necessary at a time remote from activation, thus limiting clonal expansion. Figure 3a also shows that the number of cycling B cells was greater in non-immunized mice (2-fold in TACI-positive mice and 1.6-fold in TACI-negative mice) than day 6 post-immunization. We speculate that the cycling B cells in the non-immunized mice are highly-selected QM idiotype-negative (Id^-) B cells (19) responding to a variety of environmental Ags producing diverse immunoglobulin (Ig). In contrast, after immunization with NP-Ficoll, Id^+ B cells are synchronously stimulated and produce homogeneous Ig, which will give rise to immune complexes that inhibit Id^+ B cell activation, decreasing the number of cycling B cells 6 days after immunization.

To determine the extent to which TACI decreases B cell blasts after immunization, we analyzed spleen sections and splenocytes obtained from TACI-negative or TACI-positive QM mice by flow cytometry at days 4 and 6 after immunization. Mouse B cell blasts express an epitope recognized by a rat mAb, GL7. GL7 binds to sialylated glycans, the α-2,6-linked N-acetylneuraminic acid (Neu5Ac) on lactosamine glycan chains. Neu5Ac expression increases in germinal center B cells owing to repression of CMP-Neu5Ac hydroxylase which converts Neu5Ac to N-glycolylneuraminic acid (Neu5GC) that is not recognized by GL7. Naito et al. (21) showed recently that Neu5GC represses B cell activation, and therefore specific suppression of CMP-Neu5Ac hydroxylase enhances activation of germinal center B cells. T-independent stimulation of QM mice generates short-lived germinal centers that mark extensive B cell proliferation (22, 23). The number and size of $GL7^+$ clusters was increased in TACI-negative mice 6 days after immunization (Figure 3b).

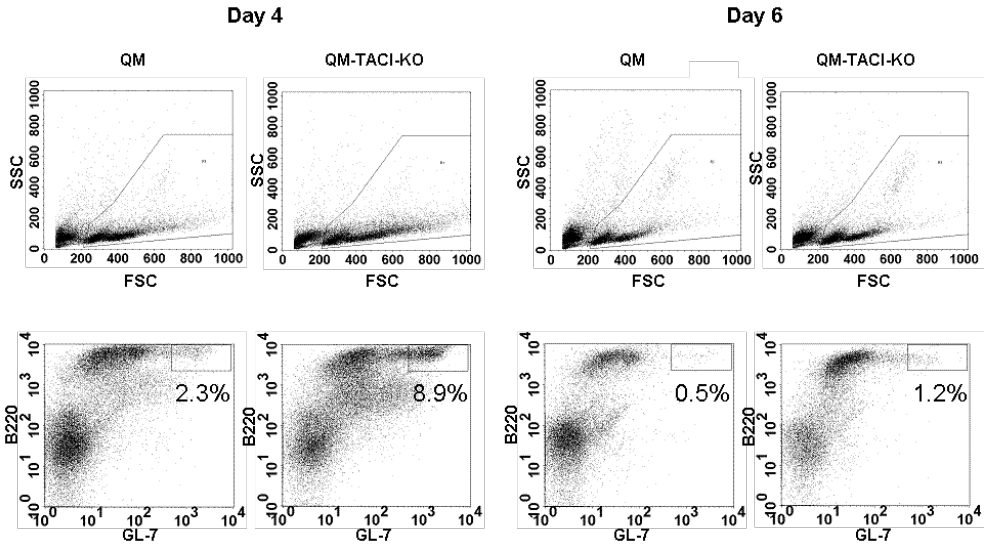
A


Basal
Day 4
Day 6
QM
QM-TACI-KO
% of cells in G2/S +M
*

B


QM TACI+/-
QM TACI-KO
GL7

C


Day 4
Day 6
QM
QM-TACI-KO
SSC
FSC
B220
GL-7
2.3%
8.9%
0.5%
1.2%

D

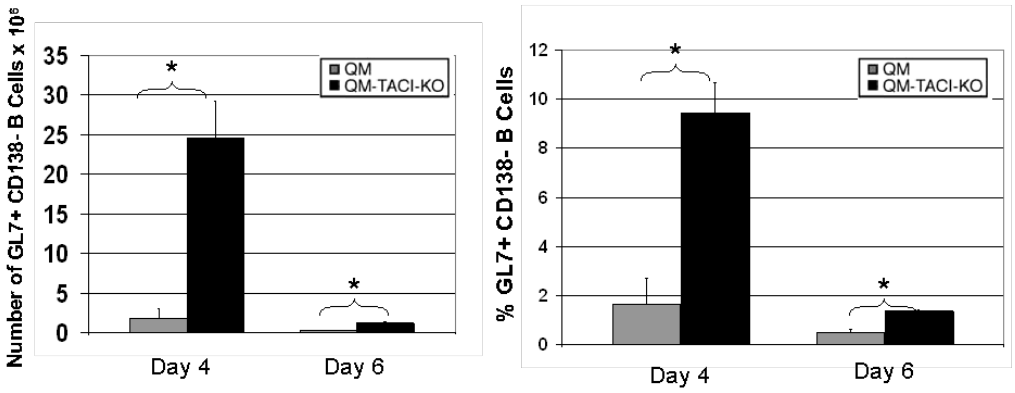

Number of GL7+ CD138- B Cells x 10⁶
% GL7+ CD138- B Cells
QM
QM-TACI-KO
Day 4
Day 6
*

Figure 3 (cont.)

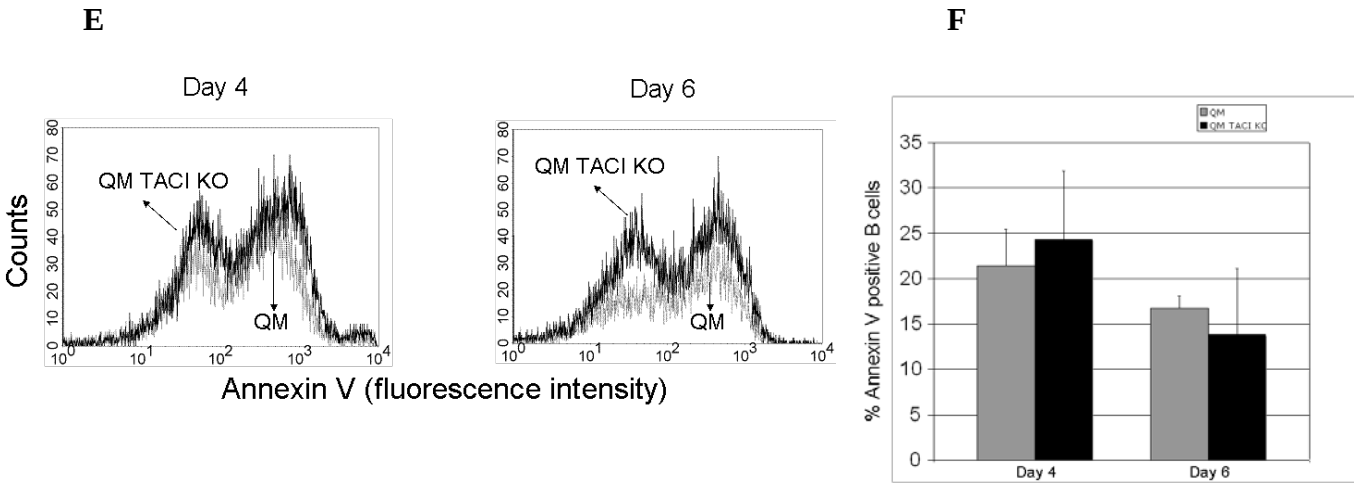


Figure 3. TACI-negative QM B cells proliferate longer than TACI-positive B cells following immunization.

(A) DNA content of $CD19^+$ B cells was determined by propidium iodide incorporation followed by fluorescence analysis with a FACScan flow cytometer and ModFiT LT software. Values are the proportion of B cells ($CD19^+$) in the G_2-S + M stage of the cell cycle at the time of immunization (basal), at day 4 or at day 6 postimmunization. Results were obtained with QM and QM-TACI-KO mice (each *n*= 3 for days 4 and 6) immunized with 30 μg of NP-Ficoll and sacrificed at the indicated times. *, Significant differences. **(B)** Spleen frozen sections obtained on day 6 after immunization, stained with anti-GL7 Abs to depict germinal centers (dark brown clusters in the follicle centers). $TACI^-$ mice have larger and more abundant clusters of $GL7^+$ B cells at the center of the follicles (germinal centers) at day 6 postimmunization and increased numbers of $GL7^+$ B cells 4 and 6 days after T-independent immunization. **(C)** and **(D)** Splenocytes were stained for GL7, CD138, and B220. **(C)** Typical flow cytometry plots representing lymphocyte distributions according to B220 and GL7 expression of gated $CD138^-$ lymphocytes. Indicated in the diagrams are the gates used to determine the absolute and relative number of $GL7^+$ B cells. **(D)** Number of $GL7^+$B cells ($B220^+$, $GL7^+$, $CD138^-$) per spleen (left) or the percentage of $GL7^+$ B cells. GL7 positivity was defined with an isotype control. Results are from three independent experiments. **(E)** and **(F)** Splenocytes were stained for annexin V, 7-aminoactinomycin D, and B220 to determine the frequency of apoptotic cells (annexin V^+, 7-aminoactinomycin D^-) after immunization. **(E)** Histograms represent annexin V fluorescence intensity of $B220^+$ cells at days 4 and 6 postimmunization. **(F)** Number of annexin V^+ B cells (annexin V^+, 7-aminoactinomycin D^-, and $B220^+$) expressed as a percentage of annexin V^+ B cells. TACI deficiency does not increase the number of apoptotic B cells in the spleen 4 and 6 days after immunization. FSC, Forward scatter.

The proportion of follicles with $GL7^+$ germinal centers was 88% in TACI-negative mice and only 51% in TACI-positive mice 6 days after immunization indicating enhanced late B cell proliferation in TACI-negative QM mice (Figure 3b). Consistently, the number and proportion of B cells (B220-positive) that are blasts ($GL7^+$) was reproducibly increased in the absence of TACI after immunization (Figure 3c, d). More than 80% of the $GL7^+$ B cells also expressed other germinal center markers such as Fas, bound the peanut agglutinin lectin, and were IgD-negative. These results suggest that TACI is necessary to stop cycling to allow terminal differentiation.

TACI-deficiency caused impaired plasma cell differentiation owing to a B cell autonomous defect

To determine whether the impaired Ab production in response to NP-Ficoll was owed to deficiency of TACI on B cells, we performed adoptive transfer experiments. Ten million QM B cells ($CD19^+$) that have or lack TACI were transferred into RAG 1-/- recipients (n=5). At day 6 post immunization spleens from recipient mice had 16 (+/- 3.9) $x10^6$ or 16 (+/- 8.9) $x10^6$ QM TACI-positive or QM TACI-negative B cells on average, respectively. Recipients of QM TACI-positive B cells produced 2789 IgM and 999 IgG NP-specific ASCs per 200.000 splenocytes, while recipients of QM TACI-deficient cells produced only 1561 IgM and 187 IgG NP-specific ASCs per 200.000 splenocytes (Figure 4). The number of NP-specific ASCs in the bone marrow was > 10-fold reduced compared with the number in the spleen and did not differ in mice reconstituted with QM TACI-positive or with QM TACI-negative B cells (Figure 4). These results indicate that expression of TACI by B cells suffices to enhance Ab production in response to TI-2 stimuli.

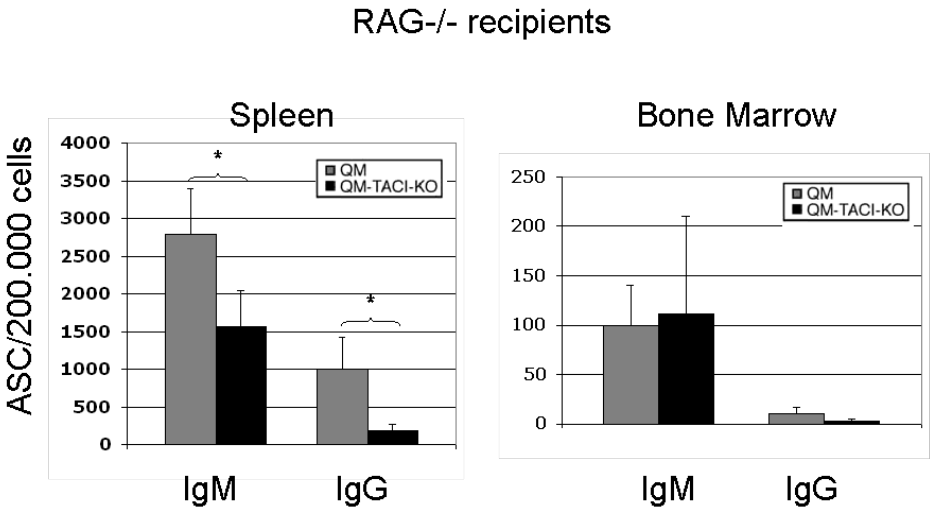


Figure 4. QM B cell autonomous TACI deficiency impairs TI-2 antibody responses.

Ten million purified $CD19^{+}$ cells obtained from the spleens of QM or QM-TACI-KO mice were transferred into $RAG1^{-/-}$ mice (*n*=5 mice per cohort). One hour later, mice were immunized with 30 μg of NP-Ficoll. The number of IgM or IgG ASCs was determined by NP-ELISPOT of splenocytes or bone marrow cells obtained from recipient mice 6 days after i.p. immunization. Recipients of $TACI^{-}$ QM B cells had only 1561 IgM or 187 IgG ASCs, whereas recipients of TACI+ B cells had 2789 IgM or 999 IgG ASCs per 200,000 B cells from the spleen. The results indicate that TACI deficiency causes a 1.8-fold decrease in the number of IgM-producing cells and 5.3-fold decrease in the number of IgG-producing cells in the spleen, 6 days after immunization. Recipients of $TACI^{-}$ B cells had 99 IgM or 10.6 IgG ASCs, whereas recipients of TACI+ B cells had 111 IgM or 2.8 IgG ASCs per 200,000 B cells in the bone marrow. *, Significant differences.

TACI-negative QM B cells were activated and proliferated as efficiently as TACI-positive QM B cells but had impaired plasma cell differentiation after stimulation in vitro.

Our findings of severely reduced serum Abs and decreased Ab-secreting cell formation in response to immunization with NP-Ficoll suggested that TACI promotes Ab production. Defective Ab production in TACI-deficient QM mice could be owed to defective B cell activation, clonal expansion or terminal differentiation. To distinguish among these possibilities, we compared activation, proliferation and differentiation of TACI-negative or TACI-positive QM B cells *in vitro*. TACI-negative B cells were activated by lipopolysaccharide (LPS) or by NP-Ficoll to the same extent and with the same kinetics as TACI-positive B cells, as indicated by CD69 expression 20 hours later (Figure 5a), indicating that TACI is not required for activation of B cells. TACI-negative splenocytes proliferated more than TACI-positive splenocytes in response to LPS but not to NP-Ficoll (Figure 5b and 5c, respectively). However, isolated TACI-negative or TACI-positive B cells proliferated equally in response to LPS (Figure 5b). Because LPS but not NP-Ficoll stimulates macrophages/monocytes and dendritic cells to produce TACI ligands (24), TACI inhibition of proliferation is only apparent in splenocyte cultures responding to LPS. It is possible that upon LPS stimulation macrophages/monocytes and dendritic cells express glycosaminoglycans that bind APRIL (25) enhancing its stimulatory function.

The inhibitory properties of TACI do not account for the notably decreased Ab responses to TI-2 stimuli. To determine whether TACI promoted plasma cell differentiation, we analyzed expression of syndecan-1 (CD138, a marker of plasma cells) by TACI-positive or by TACI-negative QM B cells by flow cytometry, 72 hours after stimulation. Figure 5d shows that the number of putative plasma cells ($CD138^{+high}$) was 2-fold lower in cells lacking TACI than in TACI-positive cells. These results confirmed that TACI is required for efficient plasma cell differentiation.

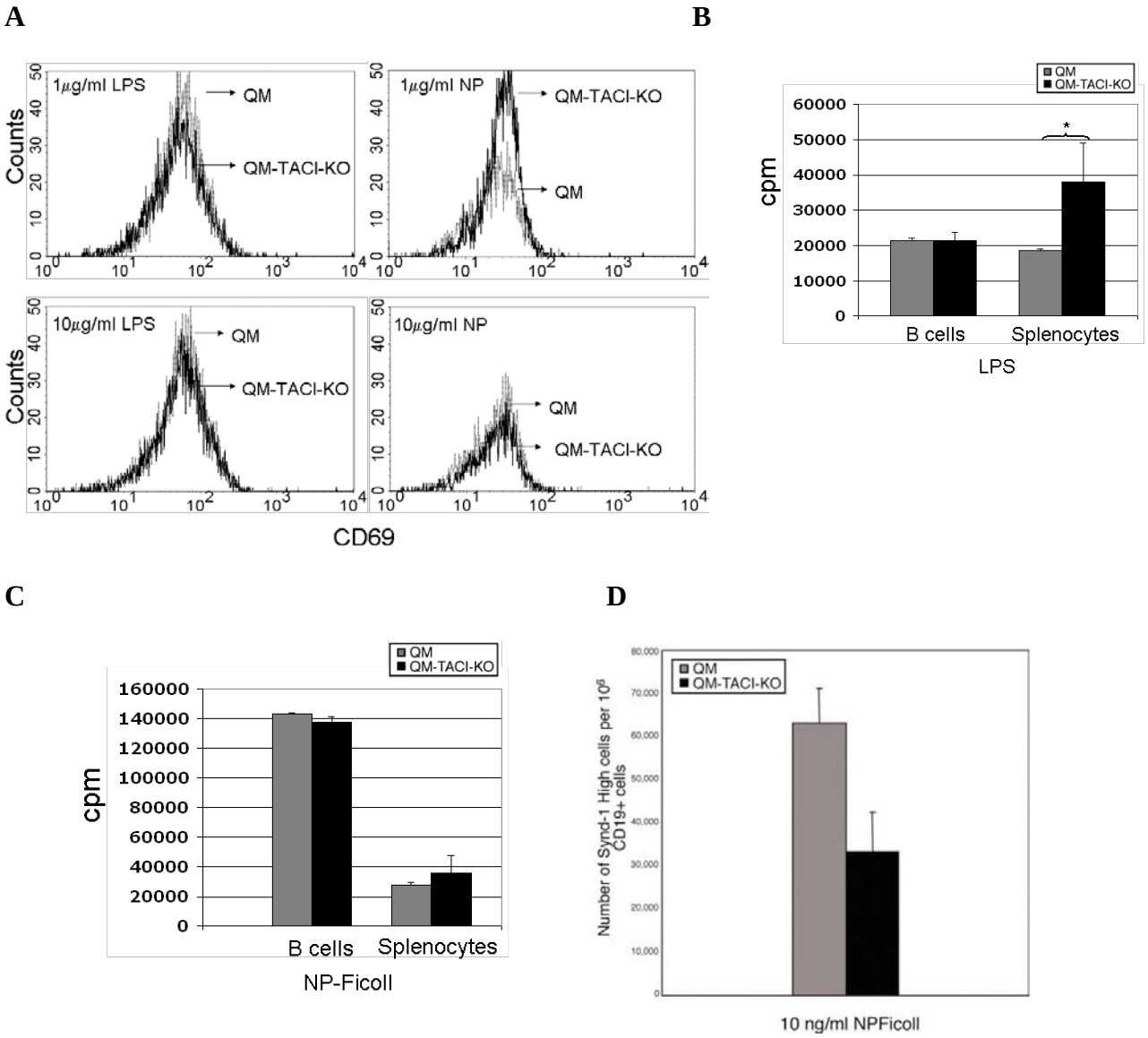


Figure 5. TACI-deficient QM B cells proliferate equally well but differentiate less than TACI-proficient B cells following stimulation *in vitro*.

(A) *In vitro* activation of QM and QM-TACI-KO B cells was assessed by surface expression of CD69 20h after stimulation of splenocytes with 1 or 10μg/ml of LPS or NP-Ficoll, as indicated. Similar results were obtained in 4 independent experiments. **(B)** and **(C)** Incorporation of [3H]TdR by splenocytes or B cells purified from the spleen cultured with LPS (0.1 μg/ml; **(B)**) or NP-Ficoll (0.1 μg/ml; **(C)**), in the concentrations indicated, for 72 h. *, Significant differences. **(D)** Number of syndecan-1^{high}, IgM^{+} in 1 million QM or QMTACI-KO splenocytes, cultured with 1 μg/ml NP-Ficoll, for 72 h. One of three experiments with similar results is shown. *, Significant differences.

Discussion

Here we show that TACI expressed by B cells has a dual function. In support of this conclusion, we found that although Ag-stimulated B cells lacking TACI were normally activated in response to Ag, they proliferated longer and differentiated poorly into ASCs both *in vivo* and *in vitro*. Thus, we conclude that a defect in plasma cell differentiation is the cause for the deficient TI-2 Ab production in TACI-KO mice.

TACI-negative QM mice produced normal levels of NP-specific IgM in response to NP-Ficoll despite severely reduced IgM ASCs. These results suggest that in QM TACI-negative mice, serum IgM is not produced by terminally differentiated B cells (detected in ELISPOT). In fact, Abs can be made by B cells before terminal differentiation in relatively small amounts on a per cell basis (26). Thus, initial IgM Ab production depends greatly on the clonal size. Because we show that TACI-negative QM B cells proliferate to a greater extent and longer than TACI-positive QM B cells the clonal size must be larger in the absence of TACI. Therefore, the relatively normal early IgM responses by QM TACI-negative mice may be attributed to Ig secretion by non-plasma cells. Because IgG-producing cells arise later, switched cells are a minority in the clone, and thus the IgG serum level is mostly the product of plasma cells. As TACI promotes differentiation into plasma cells, IgG plasma cells are decreased in TACI-negative relative to TACI-positive mice, explaining the severely reduced IgG serum levels. It is possible that accelerated proliferation of TACI-KO B cells, in fact, contributes to their decreased ability to differentiate, as is thought to occur in B cells of p18 KO mice (27).

Our results explain how human subjects with common variable immunodeficiency (CVID) owing to mutations of TACI have specific defects in the production of switched Ig isotypes with an intact class switching mechanism (28, 29). In these patients, a larger initial clonal size composed mainly of undifferentiated IgM-positive B cells would account for the relatively normal IgM levels. In contrast to IgM producing cells, B cells secreting switched isotypes are much less frequent, and therefore the serum level of switched isotypes is mostly owed to differentiated plasma cells. Thus, excessive expansion and impaired differentiation of B cells in TACI-defective subjects results in switched Ig isotype defects (28, 29).

T-independent activation of B cells induces activation induced cytidine deaminase (AID) expression (30), somatic hypermutation (23), and germinal center formation (22), and therefore may potentially originate auto-reactive Abs. How the generation of auto-reactive Abs is prevented following T-independent stimuli is not known. Assuming that AID is expressed and the rate of mutation is maintained during clonal expansion, mutants will accumulate in an exponential manner (31). Thus, increased clonal sizes owing to TACI-deficiency may increase substantially the number of B cells producing mutated and auto-reactive Abs in response to T-independent stimulation. Thus, TACI may limit the development of auto-reactive Abs by decreasing clonal expansion and because plasma cells do not mutate, by promoting rapid differentiation. Our results indicate that engagement of TACI promotes termination of proliferation and plasma cell differentiation, limiting the chances of forming auto-reactive Abs following T-independent stimulation. This possibility is in agreement with the findings of Grewal and collaborators, who showed increased incidence of autoimmune Abs in aged TACI-KO mice (17).

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Conclusions and Future Perspectives

The subject of the second part of this work was to understand how TACI promotes antibody production. In order to determine the mechanism by which TACI controls antibody responses to polysaccharides, whether and how TACI promotes QM B cell responses to a polysaccharide like T-independent antigen, the response of engineered QM TACI KO to NP-Ficoll was investigated.

It was documented that QM mice that lacked TACI produced decreased numbers of IgM (4-fold) and IgG (2.5-fold) NP-specific antibody-secreting cells (ASCs), compared to TACI-positive QM mice in response to immunization with NP-Ficoll (1). Thus, these results indicated that TACI may act at a remote time from activation since TACI was not necessary for activation and proliferation of B cells both *in vitro* and *in vivo*. Instead, TACI-deficient QM B cells remained in the cell cycle longer than TACI-proficient QM cells and had impaired plasma cell differentiation in response to NP-Ficoll (1).

In conclusion, TACI has dual B cell-autonomous functions, inhibiting prolonged B cell proliferation and stimulating plasma cell differentiation, thus resolving the longstanding paradox that TACI may have both B cell inhibitory and stimulatory functions. By promoting plasma cell differentiation earlier during clonal expansion, TACI may decrease the chances of auto-antibody production by somatic hypermutation of immunoglobulin genes in response to T-independent antigens.

These findings showing that TACI stimulates plasma cell differentiation may explain how human subjects with common variable immunodeficiency owing to mutations of TACI have specific defects in the production of switched Ig isotypes with an intact class switching mechanism (2, 3). In these patients, a larger initial clonal size composed mainly of undifferentiated IgM^+ B cells would account for the relatively normal IgM levels. In contrast to IgM-producing cells, B cells secreting switched isotypes are much less frequent, and therefore the serum level of switched isotypes is mostly owed to differentiated plasma cells. Thus, excessive expansion and impaired differentiation of B cells in TACI-defective subjects results in deficient secretion of switched Ig isotypes (2, 3).

Compromised responses to encapsulated microbes and polysaccharides in subjects with CVID owing to TACI-deficiency are compatible with B cell autonomous defects since antibody responses to polysaccharides are thought to occur independently of T cell help. It is possible that differentiation to plasma cells may also be compromised in response to protein antigens which require T cell help, explaining the serum antibody paucity in subjects with CVID owing to TACI deficiency. Alternatively TACI deficiency may impair T cell help to B cells compromising antibody response to proteins.

These data further support an important role for TACI in the differentiation of plasma cells, and its possible relevance as a target for immune based therapies not only in the context of CVID but also in autoimmunity and B cell neoplasias.

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